

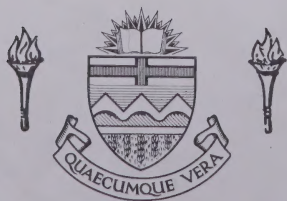
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CHEMICAL MODIFICATION OF CATALASE AND MYOGLOBIN

by



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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "CHEMICAL MODIFICATION OF CATALASE AND MYOGLOBIN" in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

The interaction of the hemoproteins horse blood catalase and sperm whale myoglobin with the pseudo-halogen cyanogen bromide (BrCN) was investigated in hopes of obtaining more information about the configuration of the active sites of these proteins.

The reaction of pretreated catalase (blocked -SH groups) and metmyoglobin with BrCN showed similarities mainly in that they were pH-invariant in the pH region of 4 to 10. On the other hand, catalase reacted faster by a factor of 4 to 5 and it gave a stable derivative, while the metmyoglobin derivative reverted readily to the native configuration.

Stoichiometric experiments showed that one molecule of BrCN was required per heme to modify both proteins, and using Br^{14}CN the incorporation of ^{14}C into the protein portions of the macromolecules was observed. Although no gross structural changes occurred, the modification resulted in significant changes in the absorption spectra of the native proteins.

Modification of catalase was accompanied by the loss of its enzymatic activity, while the ligand binding ability of both proteins was drastically reduced after BrCN treatment.

Regeneration of BrCN-metmyoglobin was found to be catalyzed by acid or base, while BrCN-catalase could be regenerated by agents such as formate, fluoride and acetate. In both cases the regenerated forms were similar to the native proteins.

Denaturation of the Br^{14}CN -modified derivatives of both hemoproteins under acid conditions resulted in the loss of label in almost quantitative amounts. The liberated H^{14}CNO was trapped as carbonate in alkaline solution.

In view of the experimental results the modification of both hemoproteins by BrCN can be most readily explained by postulating the activation of BrCN by the prosthetic group of the hemoproteins, which is then followed by the attack of a nucleophilic residue on the polarized reagent molecule.

In the case of myoglobin there is no ready alternative to histidine as the residue modified by BrCN , although some evidence is contradictory to this postulation. Experimental results obtained with catalase could also be taken as evidence that the inhibition of this hemoprotein is the result of the modification of a histidine residue. However, the drastic difference in the stability of the modified derivatives of myoglobin and catalase is not readily explained and a number of possible schemes are presented to account for it.

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CHAPTER I

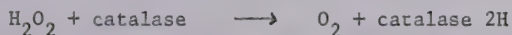
Introduction: Concepts on Catalase Mechanisms

Any discussion of the role and function of enzymes can be approached from several points of view to illustrate developing concepts of catalysis. In this respect, the studies of hydroperoxidases - catalases and peroxidases - are quite instructive and trace the evolution of our ideas on the "mechanisms" of metallo-enzymes starting from the proposition of an activated metal ion, through its enhanced functional properties due to specific metal chelation, or coordination, to the present attempts to delineate the role of apoenzyme. Indeed, the progress in understanding the catalytic features of hemoproteins has repercussions outside this important but restricted field; most recently, this is evident in shaping our proposals on the nature of enzyme allosterism of which hemoglobin is probably a prime example.

In the case of catalase, so named by Loew in 1901, the story starts with the finding by Thenard (1a,1b) - who also discovered hydrogen peroxide - that living tissues were capable of decomposing H_2O_2 . The apparently ubiquitous distribution of this enzyme, like that of sister enzymes - peroxidases - was regarded as a characteristic property of all living tissues, and their catalatic activity was likened to those of inorganic catalysts, such as platinum surfaces.

Therein lie the origins of the celebrated controversy between Wieland and Warburg which - in some measure - shaped the research on

redox reactions. Both antagonists adopted diametrically opposed views; Wieland (1c) regarded the function of redox catalyst to be the 'activation of substrate hydrogen' - which now finds its expression in our views on dehydrogenases - and assumed that in the initial redox reaction hydrogen is transferred to oxygen to form peroxide. Whilst in this he foreshadowed the discovery of oxidases, Wieland attributed to heavy metal ions only a role in the removal of peroxide. Within this framework, catalase function could conceivably be:



On the other hand, Warburg (2) disputed the formation of H_2O_2 during biological oxidations and thought that the function of heavy metal ions, particularly iron, was to activate oxygen in its direct reaction with the cell substrate; a view consistent with our present ideas on oxygenation mechanisms (3). Like Willstätter, who also doubted the function of 'iron' in peroxidases (4), Warburg did not relate catalatic reactions to the metal content of the enzyme. In this he was seemingly supported by Hennichs (5) who partially purified catalase but failed to establish proportionality between iron content and enzyme activity. Indeed, even after Zeile and Hellström (6) ascertained that catalase contains a haem compound, its role as a coenzyme was not immediately appreciated; perhaps the inertia induced by Willstätter's prestige, discouraged investigation into the chemical basis of catalysis. This outlook which apparently rated 'the unknown as more important than the known' (2) was slowly dispelled in the nineteen thirties. During that decade the protein nature of catalase was established (6); its prosthetic group was identified with protohaem IX (7) and the enzyme was crystallized by Sumner and Dounce (8). Such studies opened the way to a

systematic examination of catalase properties and led to the discovery, spectroscopic description and kinetic evaluation of its complexes with cyanide, fluoride, sulfide, formate, acetate, azide and nitric oxide (6) (9 - 14).

Simultaneously with these investigations, the physical studies of catalase were initiated and led to the determination of its hemin content (4 per molecule), sedimentation and diffusion constants ($11.3 \pm 3 \times 10^{-13}$ cm/sec. and 4.1×10^{-7} cm²/sec. respectively), partial specific volume ($0.735 \pm .005$) and the molecular weight of the enzyme ($\sim 245,000 \pm 20,000$) (15 to 21).

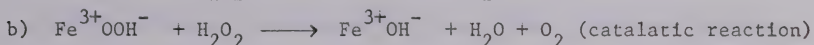
Not surprisingly, however, the issue which commanded the main interest centered on the "mechanism" of enzyme action and gave rise to three hypotheses postulating:

- a) no valency change of iron in an enzyme substrate complex
- b) free radical processes, possibly leading to "higher oxidation states" of iron, and
- c) ferrous-ferric cycles of iron valency.

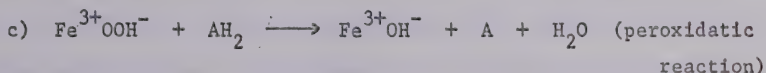
These theories, which are reviewed in some detail by Nicholls and Schonbaum (22a) were partly based on the reactions of simpler iron compounds and expressed a search for a unitary approach to the reaction of hydrogen peroxide. Thus, Stern would write: "The decomposition of hydrogen peroxide is catalysed by many agencies and we are confronted with the necessity of finding a generally applicable explanation of the reaction mechanism" (23). Some support for these views appeared to be forthcoming from various observations, notwithstanding Theorell's dictum that the role of apoenzyme must be considered in discussion of enzyme specificities (24). Stern himself (25) discovered a "red" intermediate on treating catalase with ethyl hydrogen

peroxide (now known as compound II) and attempted to correlate its formation and disappearance with the catalytic function of the enzyme. A similar entity - at least in its spectroscopic features - was discovered by Kellin and Mann (26) in reactions of horse radish peroxidase with H_2O_2 , providing evidence for "activation" of the oxidant and suggesting that the catalytic reaction represents only a special case where the hydrogen donor is a second molecule of peroxide.

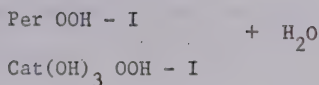
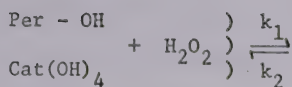
According to this view, the catalytic reaction could be written (27):



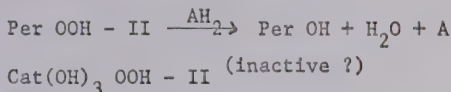
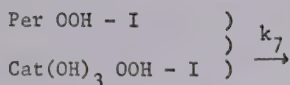
and the oxidation of ethanol to acetaldehyde mediated by catalase in presence of slowly generated H_2O_2 (28) could be also accommodated by substituting in reaction b) an hydrogen donor other than peroxide:



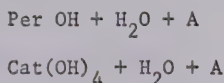
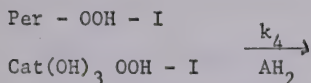
However, it was only after Chance initiated the kinetic survey of peroxidase and catalase interactions with peroxides that the intermediates postulated by Sumner et al. gained currency. Using fast reaction techniques, pioneered by Hartridge and Roughton, Chance (29) discovered the "primary" peroxide-catalase intermediate ($\text{Fe}^{3+}\text{OOH}^-$), christened compound I, and demonstrated that, unlike Stern's compound II, its activity towards hydrogen peroxide and alcohols could account for the catalytic activity of the enzyme. Extending the kinetic analysis to peroxidases (30), Chance expressed the reaction of these enzymes in a unified manner:



Primary peroxide derivatives



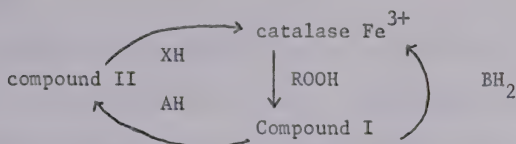
secondary peroxide derivatives



In those schemes, the requirement for different hydrogen donors, AH_2 , in the reaction mediated by catalase and peroxidase, characterized specificities of the enzyme but the inactivity of $\text{Cat(OH)}_3 \text{OOH} - \text{II}$, unlike that of $\text{Per OOH} - \text{II}$, was not explained. This constituted then but one deficiency of Chance's proposals; other concerned the ill-defined isomerisation of the primary peroxide derivative into its secondary form; the inability to account for spectroscopic changes attending the formation of compound I and the failure to explain magnetic properties of the catalase - "peroxide" intermediate (22b). Of course, the drawbacks, outlined above, do not detract from the elegance of kinetic analysis advanced by Chance. The sophistication of precise chemical interpretation attending compound I formation was not its primary purpose; it directed the attention towards more likely pathways of peroxide-enzyme interaction and stimulated vigorous interest in this area of research.

P. George addressed himself to some of these problems (31) and showed that the presumed rearrangement $\text{Per OOH} - \text{I} \xrightarrow{k_7} \text{Per OOH} - \text{II}$ represents in fact a reduction and that the resulting secondary derivative retained only one of the oxidizing equivalents of the original peroxide.

The same holds true for catalase compound II and hence the possible redox reaction can be represented as (22c):



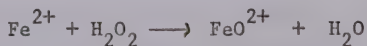
where R = H, Me, Et, Pr, Bu

$\text{BH}_2 + \text{H}_2\text{O}_2, \text{MeOH}, \text{EtOH}, \text{HCO}_2\text{H}, \text{H}_2\text{CO}, \text{HNO}_2$

AH = ascorbic acid, ferrocyanide, phenols

XH = $\text{HNO}_2, \text{HN}_3, \text{NH}_2\text{OH}$.

George (32,33) then raised the question of the unique requirement for peroxidases in generating HRP - compound I, having demonstrated that - in contrast to the corresponding catalase derivatives - it can be formed with various strong oxidizing agents such as $\text{HOCl}, \text{HOBr}, \text{NaClO}_2, \text{ClO}_2, \text{KBrO}_3, \text{KIO}_4, \text{O}_3$ and haloiridates $(\text{IrX}_6)^{4-}$. In particular, his experiments with chloroiridates were taken as evidence that compound I can be produced by electron transfer from the "ferric ion", giving rise to a higher oxidation state of iron, and possibly involving bonded oxygen in the form FeO^{3+} . In this, we see then an analogy to earlier views of Bray and Gorin (34) who postulated the formation of ferryl (quadrivalent) iron in their theory of the catalytic action of ferrous salts:



Ferguson (35) questioned George's interpretation of haloiridate - HRP reaction and suggested that the observed compound I formation was due to slowly generated peroxide; a possibility which was not given its due weight but deserves careful reconsideration in view of the observed

spontaneous reduction of hexachloroiridate (IV) in aqueous solutions (36) and the possible attendant generation of hydroxyl radical and H_2O_2 (37).

But whatever the chemical nature of compound I proves to be - whether its generation a) involves modification of the porphyrin; b) entails changes in the coordination state of iron or c) is attended by transition to a higher oxidation state of the central ion, we must explain the role of apoenzyme not only in the formation of compound I but in the stabilization of this unusual derivative and its subsequent reactions with specific reducing agents.

Rather surprisingly, there is no available data on these questions. This lack of information becomes even more puzzling, at least from the point of view of comparative biochemistry, when one recalls the remarkable functional diversity among hemoproteins containing the same prosthetic group - characteristically, ferriprotoporphyrin IX. In this class, we find myoglobin, hemoglobin; plant, yeast mold, and some mammalian peroxidases; tryptophan pyrrolase and catalase - to cite but a few examples. It is apparent, therefore, that the wide catalytic properties of these systems must stem from the differences in the prosthetic group environment within the protein matrix. And whilst such inference is intuitively plausible, the precise meaning of "differences in heme environment" remains to be translated into specific terms. Various parameters could be involved including the differences in the ligands coordinated to the coenzyme, the variation of the dielectric constant of the heme cavity, the degree of conformational freedom of the porphyrin or the changes in the nature, or steric relationships, of potential acid-base catalysts within the ligand binding radius of the transition metal.

TABLE I

Hydrodynamic Properties of Catalases

Catalase	$10^3 s_{20,w}$ sec	$10^7 D_{20,w}^2$ cm ² sec ⁻¹	Molecular weight	Investigators	References
Beef liver	11.2	4.1	248,000	Sumner and Gralen	118
Beef liver	11.295($s_{20,w}^0$)	4.17	243,000	Sund, Weber and Mülbert	119
Porcine erythrocyte	11.2	4.1	243,000	Nagahisa	42
Human erythrocyte	11.26 ^a	4.52	225,000 \pm 3,000	Stansell and Deutsch	43
Human erythrocyte	11.3 ^b			Stansell and Deutsch	43
Horse erythrocyte	11.6 \pm .2	4.15	269,000	Deutsch	21
Horse erythrocyte	11.3 \pm .1		250,000 \pm 15,000 (sed. equil.)	Ansell and Schonbaum Schonbaum	73 49
<u>Micrococcus lysodeikticus</u>	11.0		230,000	Cecil and Ogston	19
<u>Rhodopseudomonas spheroides</u>			232,000	Clayton	115

^a Enzyme obtained by $\text{CHCl}_3/\text{C}_2\text{H}_5\text{OH}$ extraction method^b Enzyme obtained without $\text{CHCl}_3/\text{C}_2\text{H}_5\text{OH}$ treatment

TABLE II

Properties of Denatured Beef Liver Catalase

Conditions of denaturation	$10^{13} s_{20,w}$ sec	$10^7 D_{20,w}$ $\frac{cm^2}{sec}$	Molecular weight	Intrinsic viscosity dl/gm	Partial specific vol. (\bar{v})	Refs.
Native	11.29	4.17	243,000	0.039(4°)	0.730	119
5 M guanidine HCl						
0.1 M mercaptoethanol	2.10	3.20	59,000			119
Succinylation	3.22	4.43	65,000			119
pH 12.65	2.89		67,000 (sed. equil.)	0.178(20°)		119
pH 12.0 (0.1 M Na_2HPO_4)	2.85		65,000 (sed. equil.)	0.23 (4°)	0.720	124
Formic acid pretreatment followed by dissociation in 20 mM sodium dodecyl sulfate	3.8	5.1	60,000			119
Performic acid oxidation followed by guanidine HCl			60,000 (?)			119

It is pertinent therefore at this point to review the present knowledge of the molecular properties of catalases. This is not intended as a comprehensive review but will cover those topics which are pertinent to the experimental work described in this thesis, and to aid the evaluation of the quality of the catalase preparations used in this study.

Chemical and Physical Properties of Catalases

Dating from the earliest studies of purified catalases, numerous investigators have repeatedly determined sedimentation and diffusion constants for the enzyme derived from various sources (Table I). Considering the diversity of preparative methods and the wide spectrum of catalase sources, the data indicates only minor variations in the hydrodynamic properties of the enzyme in spite of undoubted differences in their catalytic (115,116) and immunological behaviour (117).

The dissociation of catalase into subunits has been known since the pioneering work of Sumner and Gralen (118) and seemed to indicate a complex subunit structure contrary to the expectations based on the hemin content of the enzyme (42,120,121,122). Instructive as they are, from the methodological point of view, these studies have been lately superseded by the results of Sund et al. (119,123) whose investigations, like those of Samejima, McCabe and Yang (124) show that catalase is composed of four non-covalently linked subunits (Table II); whether these are identical remains unknown. In any case, the environments of the subunit active sites appear to be

TABLE III

The α -Helical Content of Catalases

Source of catalase	Conditions	a_o	b_o	λ_o	α -helix (%)		Refs.
					$[m']_{233}$	$\frac{CD^*}{[m']_{233}}$	
Bovine liver	pH 7	- 190	- 300	212	44	-	130
Bovine liver	pH 7					50	125
Bovine liver	Complex with cyanide, pH 7	- 200	- 260	212	27	30	125
Bovine liver	Complex with azide	- 340	- 230		27	33	125
Bovine liver	Alkali denatured	- 610	- 90	212	12	16	125
Bovine liver	Acid denatured	- 480	- 115	212	16	18	125
Bovine liver	pH 7	- 270	- 272	212			73
Bovine liver	pH 4.5 0.1 M acetate	- 270	- 261	212			73
Horse blood	pH 7	- 280	- 230	212			73
Horse blood	pH 4.5 0.1 M acetate	- 270	- 215	212			73

(Cont'd. on next page).

TABLE III

The α -Helical Content of Catalases

(Cont'd. from previous page).

Terminology: Reduced mean residue rotation $[m'] = [\alpha] \frac{m}{100} \times \frac{3}{n^2 + 2}$

where $[\alpha]$ = specific rotation; (m) = mean residue weight;

n = refractive index.

Also:

$$[m']_{\lambda} = \frac{a_{\lambda_0}^2}{\lambda^2 - \lambda_0^2} + \frac{b_{\lambda_0}^4}{(\lambda^2 - \lambda_0^2)^2}$$

where a_0 = constant representing the intrinsic residue rotation;

b_0 = -630 for a perfect right-handed helix when λ_0 is taken

as 212 m μ (126).

Calculation of helical content from CD measurements assumes that molar ellipticity at 222 m μ , $[\theta]_{222} = -37,500$ for 100% helix.

the same even if the compositions of the contributing peptide chains differ, since there appears to be no differences in the reactivity of the individual hemes of the catalase molecule with peroxides or other ligands (22a).

Although the thorough discussion of the optical rotatory dispersion and circular dichroism of catalases is beyond the scope of this work (126 to 129), it has to be pointed out that difficulties have been encountered when empirical relationships between ORD* and CD* parameters and polypeptide conformation existing in simple proteins were applied to characterize the structure of hemoproteins. For this reason a few comments will be made to provide a background for the reported ORD and CD studies on catalase.

The results listed in Table III indicate that the apparent helicity of horse blood catalase appears to be lower than the values reported for the bovine liver enzyme, providing that the empirical evaluations of presumed helicities are applicable here. That this is not the case, in spite of the support provided by studies of simple proteins such as chymotrypsin, is apparent in Dickerson's work on cytochrome c. From physicochemical measurements in solution, the helicity of cytochrome c was calculated to be 30%, in contrast to crystallographic measurements which failed to reveal evidence for any α -helical region. This finding might be taken as an indication that the optical activity, which is usually associated with the peptide

* Abbreviations used are: ORD, optical rotatory dispersion; CD, circular dichroism.

bonds in α -helical segments, might originate from other interaction(s). This contention is well supported by the fact that reduction causes marked changes in the ultraviolet CD spectra of cytochrome c and of the heme undecapeptide derived from cytochrome c (169). Obviously, the CD changes in the case of the heme undecapeptide cannot be due to conformational changes, but may be partly caused by a change in the optical activity of the heme or possibly of the coordinated histidine.

Breslow and coworkers argued that the optically active heme transitions do not affect the amplitude of the 225 m μ Cotton effect observed in metmyoglobin, because this ORD band remains constant when the protein is converted to its azide and hydroxide derivatives (170). On this basis they assigned the changes observed in the ORD and CD bands in the 220 to 235 m μ region upon the removal of heme from metmyoglobin to the unfolding of an α -helical region of the protein.

Strichland et al. observed similar changes in the far ultraviolet CD bands (at 191,208 and 222 m μ) of horse radish peroxidase when the heme was removed from the enzyme (171). They argued that, although these bands are associated with peptide transitions, a change in their intensities should not be taken as an indication of conformational changes of the polypeptide chain without careful consideration, because interactions between the heme and the peptide bonds, such as electric-electric and electric-magnetic dipole moment couplings, could easily influence the intensities of the far ultraviolet CD bands (172,173,174). The contribution of these interactions to the far ultraviolet CD spectra of hemoproteins was not estimated.

TABLE IV

Amino Acid Composition of Catalases^a

Amino acid	Source of catalase		
	Beef liver ^b	Horse liver ^b	Rat liver
Ala	154	157	169
Arg	130	121	126
Asp	294	266	286
Cysteine	-	-	8 ^d
Half cystine	29	-	12
Glu	197	213	231
Gly	155	154	149
His	89	70	81
Ileu	79	85	84
Leu	154	162	137
Lys	116	126	131
Met	41	44	52
Phe	132	120	128
Pro	166	167	176
Ser	97	104	105
Thr	96	107	108
Try	-	-	33
Tyr	86	80	80
Val	141	156	148

^aNumber of amino acid residues per mole of catalase.^bData of Schroeder et al. (57).^cData of Higashi et al. (75).^dDetermined by PCMB titration.

In view of these findings, the assignment of absolute α -helix content to catalases is not readily justified. Furthermore, the ORD and CD changes observed when catalase is converted to its azide and cyanide derivatives (125) should not be definitely interpreted as structural changes. The absence of such changes in the case of the acetate derivative of catalase supports this contention.

Clearly, this subject demands a detailed study of the possible interactions of the heme with the polypeptide chain before our knowledge about the optical activity and the conformation of simple proteins can be applied to hemoproteins with confidence.

The development of relatively rapid procedures for the separation of amino acids (55) and the increasing availability of highly purified proteins led to the evaluation of amino acid composition of various enzymes (81) - a step which is a prerequisite to the subsequent determination of the primary or even tertiary structures.

Such data is not available for horse blood catalase - except for Bonnichsen's (40) tentative estimate of arginine, lysine, histidine, glutamic and aspartic acid. On the other hand, the composition of beef, horse and rat liver catalases have been examined in several laboratories (82,83,85) and the results of these investigations, are summarized in Table IV.

The amino acid analysis of beef erythrocyte catalase, reported recently by Deisseroth and Dounce (84), is not included in Table IV, because the values were given in a unit (no. of amino acid residues/100 total amino acid residues) which cannot be converted, with any

confidence, to the unit used in Table IV. Nevertheless, their comparative table shows a reasonable agreement between the amino acid compositions of beef liver and beef erythrocyte enzymes.

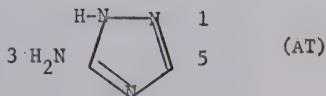
A number of amino acid functional groups have been implicated to take part in the catalytic function of catalase. Available data pertinent to this issue will be discussed briefly.

Unfortunately, as in most other investigations of catalases, the tryptophan content remains to be established; judging however from Schroeder's results (83) and from the peptide map analyses, its level is not less than 24 in beef liver enzyme. Whether tryptophan plays any catalytic role is still debatable, but its involvement was challenged by Nakatami (86). He noted that the decrease in catalase activity, following photooxidation, is not reflected in tryptophan destruction, although several other amino acids were degraded - principally, histidine, tyrosine and methionine. Since this, in turn, must lead to an alteration of enzyme structure and a possible impairment of catalatic activity, the results of photooxidation studies, like those involving gross carboxymethylation of the enzyme (87), do not permit any influence bearing on the role of a given amino acid moiety.

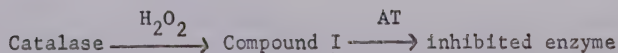
Using kinetic analysis proposed by Koshland et al., (88) Nakatami demonstrated some parallelism between the rate of destruction of "slow" histidines and the decrease of catalase activity, and proposed that histidine(s) might influence the heme-protein interaction. This generalization, not entirely justified on experimental basis, proved to be an inspired guess, but due to the extensive modification

of the protein the specific role of histidine(s) remained undefined. For example, this versatile residue could function as a) a distal ligand (i.e. as a group bonding at the fifth coordination position of iron-porphyrin complex); b) a counter ion to the "propionic acid" side chains of hemin (89) or c) a general acid-general base catalyst (90). Equally, different histidines may perform, simultaneously, functions indicated in a), b), and c).

Partial answers to these problems began to emerge in the course of this work and partly became apparent in the investigations of Margoliash, Novogrodsky and Schejter (91,92,93), who following the observation by Heim, Appleman and Pyfram (94) that 3-amino-1,2,4 triazole (AT) irreversibly inhibits catalatic function in liver



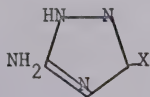
but not in the purified enzyme, noted that the inhibition depends on the presence of peroxide and proposed a reaction between catalase compound I and AT.



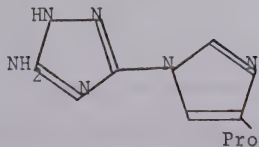
In their initial interpretation, Margoliash et al., (93) envisaged a nucleophilic attack by AT on a residue X situated in the vicinity of hemin; in later studies, using horse liver catalase, X was shown to be "histidine-74" situated in the sequence: Val - Val - His - Ala -

Lys (95). Interestingly enough, a similar peptide is also obtained upon tryptic cleavage of bovine liver catalase (57) - an indication of significant homologies among catalases derived from different species.

The course of chemical events leading to inhibition of catalase by 'AT' is still in dispute. On one hand, Schonbaum prefers the pathway involving oxidation of amino-triazole by catalase compound I generating an entity susceptible to nucleophilic attack by imidazole ring of HIS-74 (96). An opposite viewpoint is held by Margoliash who postulates an interaction between HIS-74 and the unmodified AT, implying that ... "the residue to which AT is bound must be specifically activated in catalase - $\text{H}_2\text{O}_2\text{-I}$..." (97). Furthermore, his evidence seems to contradict Schonbaum's expectation of aminotriazole substitution at C_5 to give



or specifically



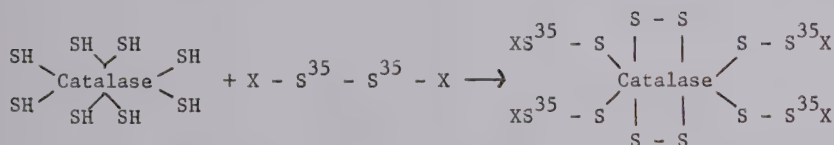
(assuming $\text{X} = \text{HIS}$)

since, according to preliminary structural studies, an N - N bond is formed between AT and HIS.

The chemical analysis of aminotriazole - compound I reaction is immediately pertinent to the hypothesis bearing on BrCN - catalase interaction and it will be argued that Margoliash's proposals are

not tenable providing that both reactions entail modification of the same residue (see Chapter VI).

Apart from the studies of the potential role of histidine(s), the possible involvement of cysteine and cystine engaged the attention of several groups of investigators. The work in this field was initiated by Pihl and Eldjarn (98,99) who demonstrated that beef liver catalase contains reactive SH groups since treatment with cystamine, $S_2(CH_2CH_2NH_2)_2$, led to the formation of mixed disulfides. This facet of catalase reactivity was later exploited to show that spatial relationships of cysteine residues permit the formation of intramolecular S - S bonds (100). Thus, reaction of catalase with radioactive cystine led to the modification of eight -SH groups, simultaneously with the incorporation of four S^{35} labelled cysteines, as expressed schematically in Eq. 1:



Equation 1.

As might be expected, studies on modification of sulphydryl groups were not limited to interchange reactions but were extended to include mercaptide formation - generally, using "p-chloromercuribenzoate" (101, 102,75). Other traditional -SH reactants, such as iodoacetic acid, iodoacetamide (103), acrylonitrile, ethyleneimine, N-ethyl maleimide or 5, 5'-dithio-bis-(2-nitro benzoic acid), although of interest whenever the formation of stable sulphydryl derivatives is desirable, proved less useful in the assay reactions (49).

TABLE V

Sulphydryl and Half-cystine Content of Catalases^a

Source of catalase	-SH group determination			Half-cystine determination		
	No. of -SH groups		Method used	Conditions of denaturation	No. of half-cystines ^b	Method used
	Native	Denatured				
Bovine liver	8	-	Disulfide interchange; pH 7.4	-	-	- (98)
Bovine liver	6	10	PCMB and Ag ⁺ titrations	8 M guanidine	-	- (101)
Bovine liver	6	16	PCMB titration	Acid, pH 3	-	- (105)
Bovine liver	7 ± 2	-	Hg ⁺ titration, pH 8.5	-	19 ± 4	Ag ⁺ titration in presence of sulfite, pH 8.5 (103)
Bovine liver	-	-	-	-	16 - 17 ^c	Cysteic acid determination (57)
Bovine liver	-	-	-	-	16 (minimum)	Amino acid sequence (83)
Bovine liver	-	-	-	-	9	Amino acid analysis (84)
Rat liver	8	-	PCMB titration	-	20	Amino acid analysis (75)

TABLE V (Cont'd.)
Sulphydryl and Half-cystine Content of catalases^a

Source of catalase	-SH group determination		Half-cystine determination	
	-SH groups		Conditions of denaturation	No. of half- cystines ^b
	No. of Native	-SH groups Denatured	Method used	Method used
Rat erythrocyte	-	-	-	20
				Amino acid analysis
				(75)
Rat erythrocyte	-	-	-	21 ± 1
				Amino acid analysis
				(84)

^aNumber of residues per catalase molecule.

^bSum of cysteine and half-cystine residues.

^cNot corrected for hydrolytic destruction.

These enquiries led to a consensus - from which only Hermel and Havemann (103,104) partly dissent - that:

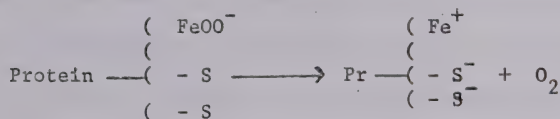
- a) the activity of the enzyme is not dependent on the viability of all -SH groups;
- b) some sulfhydryl groups are masked;
- c) the physical characteristics of the enzyme - optical spectra, between 350 and 700 mμ, and hydrodynamic properties - do not reflect -SH alkylation, oxidation or mercaptide formation.

Nonetheless, in spite of repeated attempts, the estimate of cysteine and cystine residues still remains tentative, as is apparent from the survey in Table V.

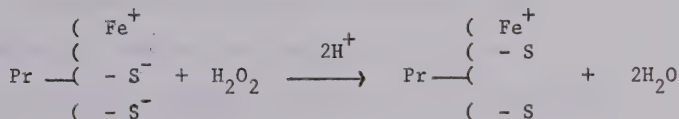
Comparison of sedimentation patterns of fresh and stored enzymes revealed slow formation of a higher molecular weight species (49,43,18). More definite indications that the alteration of catalase properties could result from partial oxidation of sulfhydryl groups are apparent in the investigation of Cantz (108) and Heidrich (109,110) who demonstrated chromatographic and electrophoretic homogeneity of catalases prepared either in the absence of oxygen or in the presence of reagents which prevented -SH oxidation (dithiothreitol, iodoacetamide). Significantly, such homogeneity is not maintained in the presence of air, unless the enzyme is pretreated with an SH protecting agent, e.g. iodoacetamide.

Hemin degradation products, which are almost invariably present in liver enzymes, could also influence the sulfhydryl content of liver catalases by oxidation of -SH groups to cystine residues.

This problem reflects then still another facet of possible structural relationships between cysteines or cystines and the prosthetic group, and found expression in the mechanism of catalatic reaction proposed by Hermel and Havemann (104). In a remarkably bold scheme, which invokes atypical property of disulfide systems (111), they envisage the reactions outlined in Equations 2 and 3.



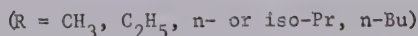
Equation 2.



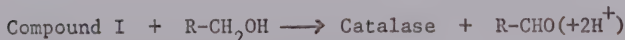
Equation 3.

but failed to explain:

- 1) the formation of catalase compound I with alkyl peroxides;



- 2) the peroxidatic function of catalases



- 3) pH-invariance of catalatic or peroxidatic activity
(110,113)
- 4) the spectroscopic and magnetic properties of enzyme
peroxide derivative (22).

Francoeur-Denstedt reaction. In addition to the well known catalytic functions of catalase, catalatic and peroxidatic activities, it has been found that the enzyme can also enhance the oxidation of ribose-5-P by ferricyanide in mildly alkaline solutions (114). Francoeur and Denstedt postulated that catalase-Fe³⁺ becomes reduced by the sugar phosphate and then the resultant catalase-Fe²⁺ is oxidized by ferricyanide to regenerate the catalyst (114). However, Margoliash and Novogrodsky showed (91) that, although AT-treatment of catalase had resulted in about 96% inhibition of its catalatic activity, the ability of the enzyme to mediate the oxidation of ribose-5-phosphate in the presence of ferricyanide was not altered to a significant extent. This finding appears to indicate that the oxidation of ribose-5-phosphate in the presence of ferricyanide is not catalyzed by the heme group and its immediate environment, but rather by another portion of the protein molecule that is not involved in the catalatic mechanism.

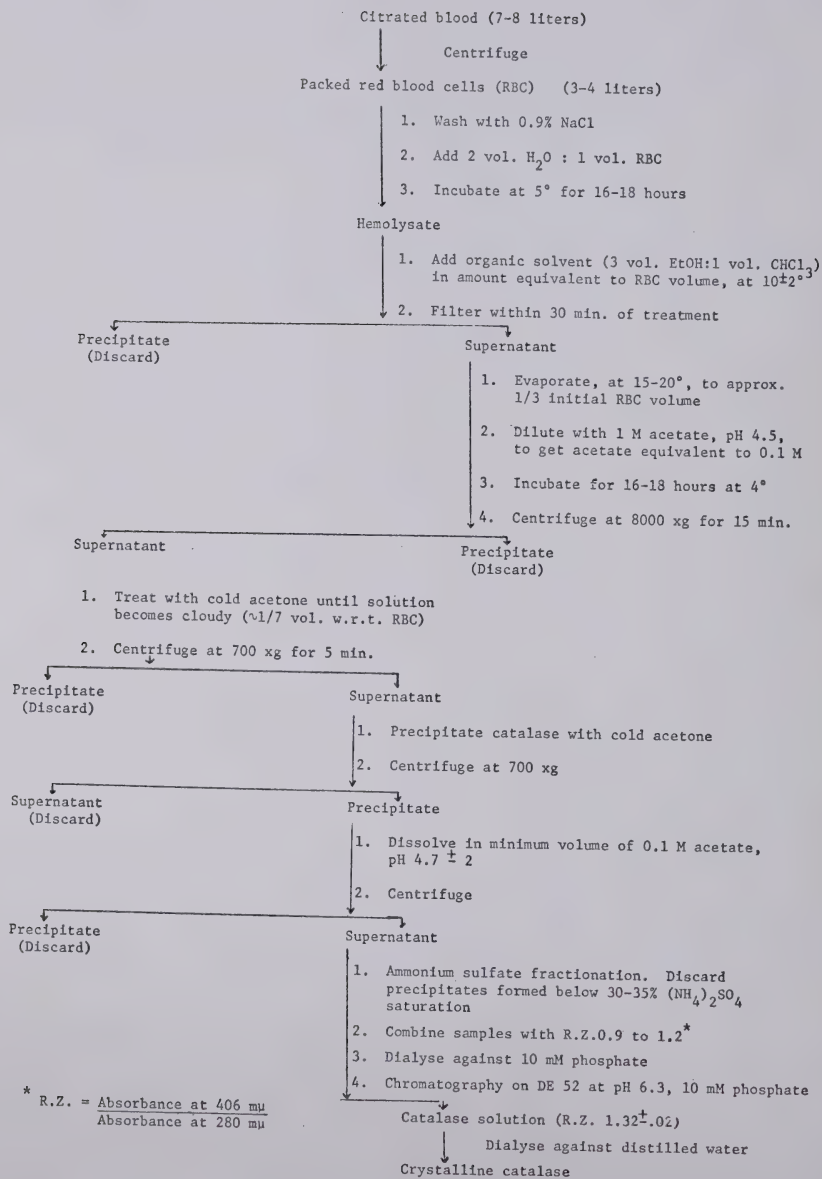


Fig. 1: Flow chart of the purification procedure of horse erythrocyte catalase.

CHAPTER II

Materials, Methods and Instrumentation

This chapter deals with the general experimental methods and will review:

- a) Preparation of biological materials
- b) Preparation and assay of reagents
- c) Physical methods of analysis.

Isolation, purification and crystallization of horse erythrocyte catalase

Catalase ($\text{H}_2\text{O}_2:\text{H}_2\text{O}_2$ oxidoreductase EC 1.11.1.6) was one of the first enzymes to be isolated in a high state of purity and its crystallization from bovine liver (38) ranked among the early successes of biochemistry. The enzyme, derived from various species, is characterized by a relative stability in presence of organic solvents such as ethanol, chloroform, dioxane and acetone; it is precipitated from aqueous solutions by 45-60% saturation with ammonium sulfate; it is adsorbed on DEAE cellulose at low ionic strength, pH 7.5, and is eluted therefrom by increasing ionic gradient. These properties, then, form the basis of the traditional preparative methods (39-43). Nonetheless, to ensure optimum yields of a viable product, the isolation of the enzyme, from different species, necessitates procedural variations. The methodology used in this work is outlined in the accompanying flow chart (Fig. 1).

The main differences in this procedure compared to the published methods lie in the adoption of an empirical approach. For example, the separation of catalase from erythrocyte hemoglobin, was achieved, following ethanol-chloroform treatment, by exposure of red cell lysate for a minimum time necessary to precipitate hemoglobin (generally less

than 30 minutes) rather than by incubating the reaction mixture for any prescribed period which, in the hands of other investigators, ranged from 1/2 to 3 hours. In addition, it was noted that superior yields of the enzyme, characterized by a higher specific activity, could be obtained by adopting the following precautions:

- a) the temperature of cell lysate should be maintained at approximately 8° in the course of addition of organic phase;
- b) mixing of the organic solvents with the lysate should be carried out stepwise, over 5 - 10 minutes, under constant and vigorous stirring;
- c) following treatment with ethanol-chloroform, the reaction should be maintained between $12 - 15^{\circ}$;
- d) separation of the supernatant, containing catalase, and subsequent stripping of organic solvent from the filtrate must be performed below 20° ;
- e) in the course of precipitation of catalase with acetone it is advisable to maintain the temperature at $0 \pm 2^{\circ}$;
- f) protein precipitating below 30% ammonium sulfate saturation, at pH 4.7, should be rejected;
- g) final purification of catalase, characterized by $A_{406}/A_{280} \sim 1.3$ can be achieved on chromatography with DEAE cellulose (Whatman DE - 52) at pH 6.3 in the presence of 10 mM phosphate. Under these conditions, the contaminating protein are preferentially retained on the column, permitting isolation of the enzyme which readily crystallizes on dialysis against distilled water. Its physical and chemical properties will be described in the following chapters.

A batch of 7 - 8 liters of horse blood usually yielded 200 to 800 mg catalase, as it was estimated before the final purification step or column chromatography. By carefully observing the outlined precautions yields of 500 mg and more were obtained consistently during the later part of the work. Column chromatography and crystallization, of course, resulted in losses of up to 40% of the crude material.

Chemicals

Chemicals used throughout this work were of the highest quality available commercially and unless otherwise specified were not further purified. Aqueous solutions were made habitually in water distilled from alkaline permanganate.

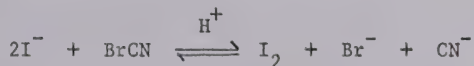
Purification and assay of cyanogen bromide

Cyanogen bromide, supplied by Eastman Kodak (White Label), was frequently contaminated by an impurity which was ascribed to the polymerization of pseudo-halogen. However, since BrCN readily sublimes, even at 50° - in contrast to its polymerized derivative - the purification of cyanogen bromide was achieved by static distillation at 2 - 5 mm (Hg) pressure. To prevent polymerization, or decomposition of the product, the purified BrCN was stored, at -10°, or dissolved in an inert solvent, for instance - dry acetonitrile.

An aqueous solution of C¹⁴ labelled BrCN (sp. activity 2 mc/m mole) was obtained from Merck (Canada). In acid solution, at pH not greater than 5 and at 0° - 5°, cyanogen bromide does not hydrolyse appreciably, as was noted by Schreiber and Witkop (44), Møller (45) and Griffith (46).

Assay of cyanogen bromide

Several methods are available for the estimation of cyanogen bromide and exploit different facets of its oxidizing potential. Of these, the oxidation of iodide to iodine:



is readily accomplished and permits the quantitative estimation of BrCN by titrating liberated iodine with thiosulfate. The solution of sodium thiosulfate was standardized - in a traditional manner - against acidified potassium iodate in the presence of excess iodide (47).

Assay of C¹⁴ labelled cyanogen bromide

Whilst the iodometric procedure is the method of choice for the estimation of 0.01 to 0.1 M BrCN, and polarographic assays can be used at 10^{-4} to 10^{-3} M level of the reagent (48,49), it was felt necessary to resort to a more sensitive assay in the estimation of the radioactive material. Such a method was developed by Murty and Viswanathan; it depends on the formation of a coloured product in presence of pyridine, barbituric acid and cyanogen bromide (50). The product, whose chemical composition has not been determined, is characterized, at 587 m μ by high molar extinction coefficient ($\epsilon_{587} = 9.24 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) - thus permitting an accurate estimate, even of 1 μM BrCN. In the assay, 1 ml of the reagent - which consists of 6.7 M pyridine, 0.77 M HCl and 0.19 M barbituric acid - was added to 3.0 ml of aqueous cyanogen bromide, the reaction vessel

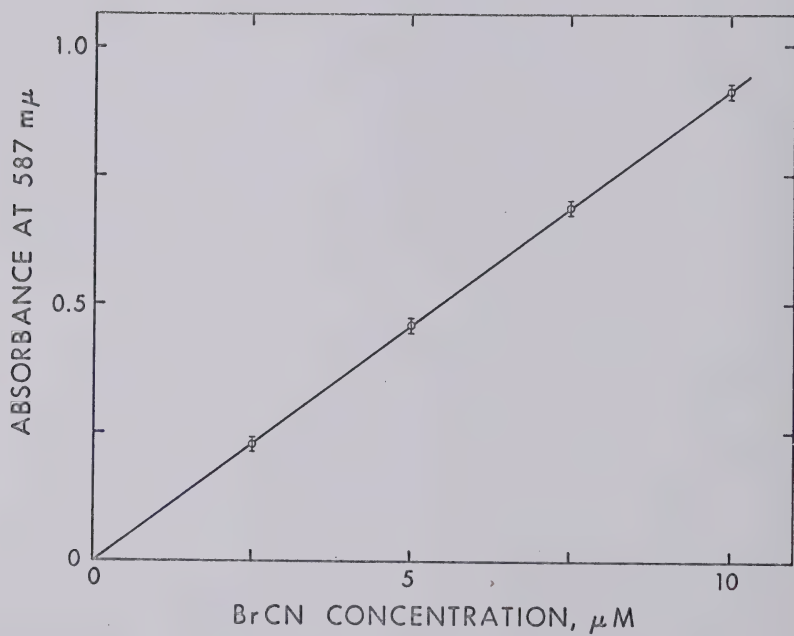


Figure 2. Spectrophotometric estimation of BrCN.
Final concentrations in reaction mixture.

tightly stoppered and the reaction mixture incubated at 40° for 45 \pm 5 min. During this period, the colour intensity reaches a maximum level and remains steady for at least 20 min.

Of course, before resorting to this procedure it was shown, using standardized BrCN, that the resulting absorbancy is directly proportional to the initial BrCN concentration. The results are summarized in Fig. 2.

Potassium cyanide

Solutions of potassium cyanide were titrated with silver nitrate, according to standard procedure (51). Since on prolonged storage some evaporation, or polymerization, of HCN is unavoidable it is advisable to prepare fresh solutions whenever required.

Ammonium bromide

Bromide (\sim 0.1 M) was assayed against silver nitrate using potassium chromate as indicator (52). The appropriately diluted solutions of ammonium bromide were subsequently subjected to neutron activation analysis; the experimentally determined levels of Br⁻ and the calculated values were found to be in excellent agreement (see p. 53).

Assay of 2-mercaptoethanol

To prevent rapid autooxidation of the thiol, the stock solutions of 2-mercaptoethanol were prepared in cold, oxygen-free water previously acidified with sulfuric acid; under these conditions, the thiol appears to be stable for at least one week. Its concentration, \sim 0.05 M, was estimated by oxidation with an excess of standardized iodine and back

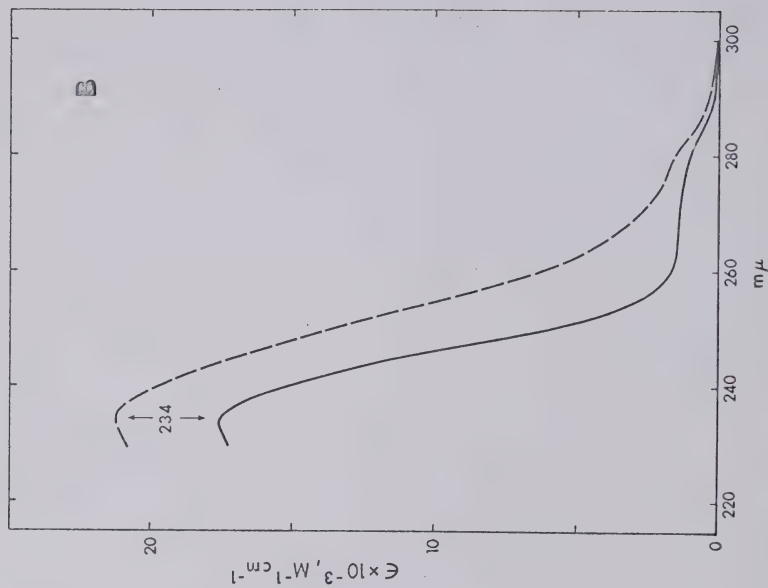
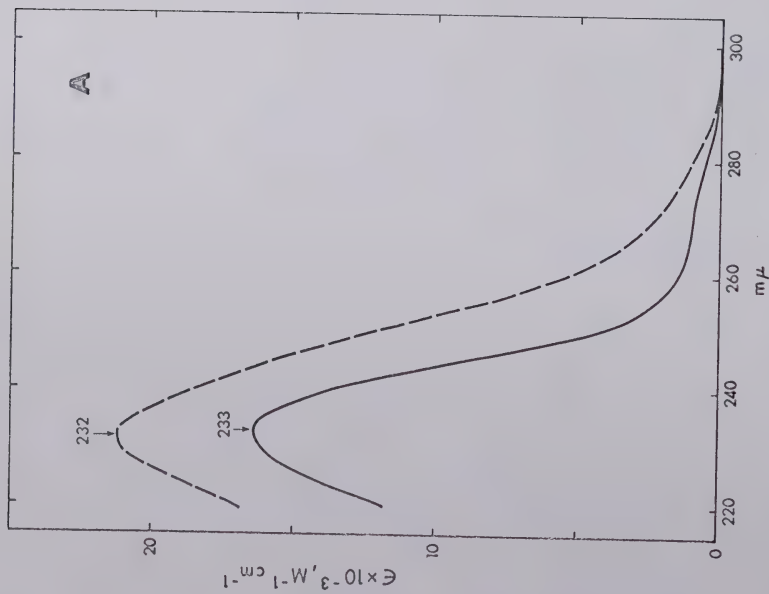


Figure 3. Ultraviolet absorption spectra of PCMB and its mercaptide with 2-mercaptoethanol.
 A: 0.05 M phosphate, pH 7.0, 25°. B: 0.33 M acetate, pH 4.6, 25°.
 PCMB: —; mercaptide: - - -.

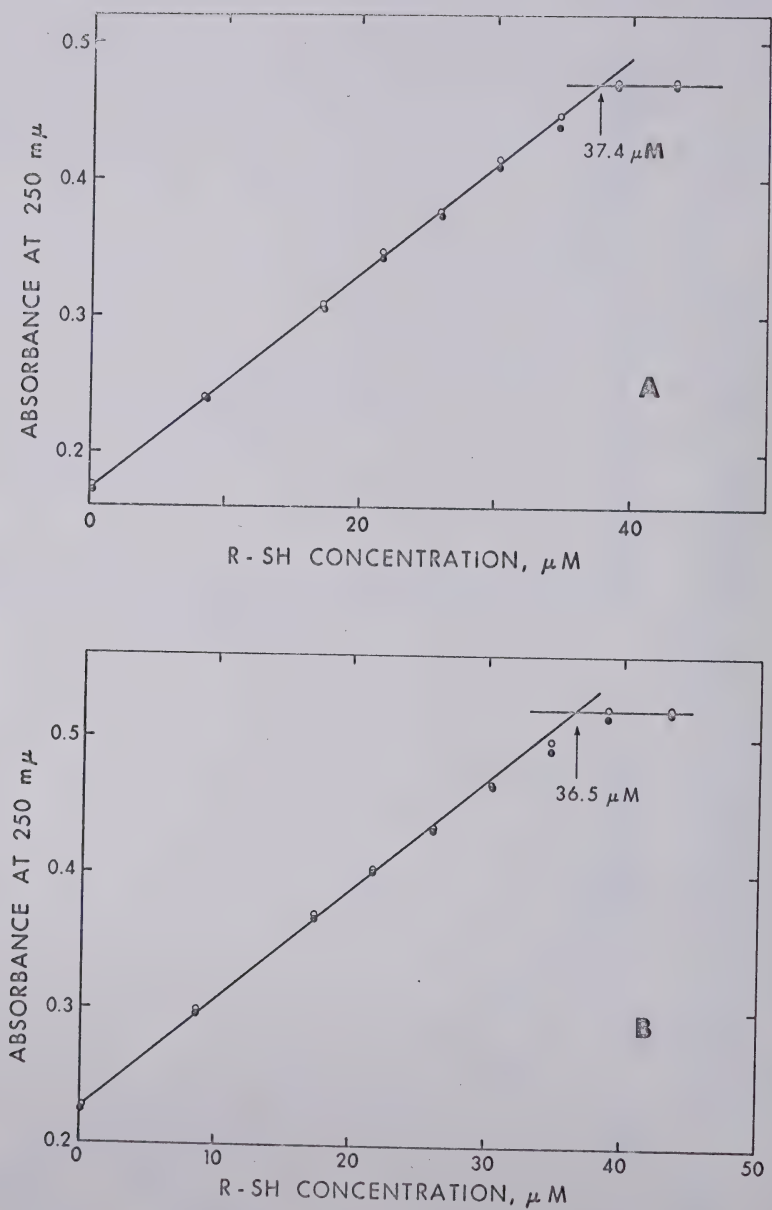


Figure 4. Spectrophotometric titration of PCMB with 2-mercaptoethanol, $37.5 \mu\text{M}$ PCMB. A: 0.02 M phosphate pH 7 (\bullet), 0.2 M phosphate, pH 7 (\circ). B: 0.1 M acetate, pH 4.6 (\bullet), 0.33 M acetate, pH 4.6 (\circ).

TABLE VI

Extinction Coefficients of PCMB, $\epsilon \times 10^{-3}$, $M^{-1} \text{ cm}^{-1}$

Wavelength, m μ	270	260	255	250	240	234	233	230
Acetate, This work ^a	-	1.67	2.89	5.92	15.15	17.50	-	17.20
pH 4.6 Boyer's ^b	-	1.91	2.89	5.72	15.45	17.18	-	16.32
Phosphate, This work ^c	1.00	-	2.41	4.61	13.70	-	16.60	16.30
pH 7 Boyer's ^d	1.01	-	2.54	4.84	14.15	-	16.55	16.40

Extinction Coefficients of the Mercaptide of PCMB, $\epsilon \times 10^{-3}$, $M^{-1} \text{ cm}^{-1}$

Wavelength, m μ	270	260	255	250	240	234	233	230
Acetate, This work ^a	-	6.37	9.79	13.70	19.65	21.20	-	20.80
pH 4.6 Boyer's ^b	-	5.61	9.17	13.40	19.78	20.90	-	20.10
Phosphate, This work ^c	2.36	-	8.51	12.30	18.50	-	21.20	21.20
pH 7 Boyer's ^d	1.69	-	8.23	12.19	18.62	-	20.55	20.50

Changes in Extinction Coefficients Upon the Formation of the Mercaptide

of PCMB, $\Delta\epsilon \times 10^{-3}$, $M^{-1} \text{ cm}^{-1}$

Wavelength, m μ	270	260	255	250	240	234	233	230
Acetate, This work ^a	-	4.70	6.89	7.69	4.35	3.70	-	3.54
pH 4.6 Boyer's ^b	-	3.70	6.28	7.68	4.33	3.72	-	3.78
Phosphate This work ^c	1.37	-	6.10	7.70	4.82	-	4.66	4.97
pH 7 Boyer's ^d	0.68	-	5.69	7.56	4.48	-	4.00	4.12

^a 0.33 M acetate, pH 4.6, 25°; 38 μM PCMB

^b From Fig. 1 of Ref. (53); 0.33 M acetate, pH 4.6

^c Phosphate buffers, 0.02 - 0.2 M, pH 7, 25°; 38 μM PCMB

^d From Fig. 2 of Ref. (53); 0.05 M phosphate, pH 7.0.

titration of unreacted iodine with thiosulfate (47). In view of the close agreement between the expected and experimentally determined values, the oxidation of the alcoholic moiety of the thiol apparently does not occur.

p-Chloromercuribenzoate (PCMB)

Approximately 4×10^{-3} M solutions of 'Sigma' PCMB were prepared in water alkalized with sodium hydroxide. The solution was then titrated with standard mercaptoethanol according to the general principles outlined by Boyer (53). The method exploits absorbancy changes, below 290 m μ , which accompany the formation of mercaptide, as shown in Fig. 3 and Table VI.

The titration of PCMB with 2-mercaptoethanol was carried out in phosphate buffers at pH 7, and in acetate buffers at pH 4.6. It was found that the concentration of the phosphate buffers did not influence the results of the titrations, see Fig. 4A; on the other hand some difficulties were encountered in acetate buffers. The main problem appeared to be that endpoints of the titrations were significantly different from those obtained in phosphate buffers; in addition the endpoints varied as the concentration of acetate was changed. Some of these problems are illustrated in Fig. 4B. In general, the results obtained in phosphate buffers were much more reliable, and therefore this buffer was used for the routine estimation of PCMB by 2-mercaptoethanol titration.

Table VI provides a comparison of the spectral properties of PCMB and its mercaptide as given by Boyer (53) and as they were found during this investigation. The differences observed could be

due to the fact that Boyer used cysteine to form the mercaptide, while in these studies 2-mercaptoethanol was utilized for the same purpose.

Physical methods of analysis

1. Spectrophotometry. The measurements were carried out at controlled temperatures using either the Cary 14, Beckman DK-2 or Beckman DU equipped with the Gilford optical conversion unit. The reading on the Cary 14 served as a reference standard for studies with other instruments.
2. Circular dichroism (CD). The spectra were determination using the Cary 60 Spectropolarimeter, equipped with a Cary Model 6001 circular dichroism attachment.
3. Measurement of radioactivity.

Liquid Scintillation counting. All radioactivity assays were made with Nuclear Chicago, Series 725, controlled temperature, spectrometer system. The performance of the instrument was established against standard (NBS) C^{14} -labelled n-hexadecane, using Bray's solvent (54). The effect of various experimental samples on the quenching of scintillation emissions was then determined in the presence of the calibrated C^{14} compound; and, from the observed "quenching efficiency" the amount of C^{14} in the unknown samples could be calculated.

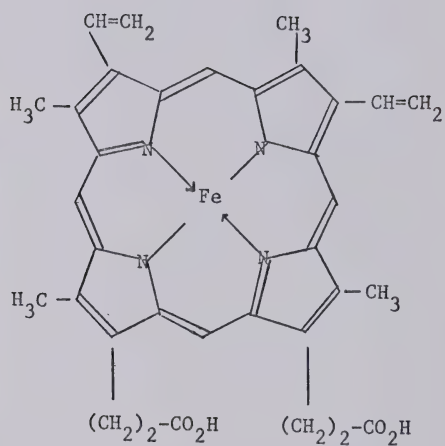
Scintillation medium. Unless otherwise stated, 10 ml of scintillation solvent was used in all experiments. The

medium was prepared according to Bray, except that ethylene glycol was not incorporated in the scintillation fluid (54).

Paper strip counting. The location and estimation of C^{14} on paper chromatograms were accomplished by using a Nuclear Chicago Actigraph III. The instrument was calibrated with a paper strip on which known amounts of C^{14} -labelled protein had been spotted.

4. Potentiometry. pH of the reaction media were determined with Radiometer pH-meter 25 and Radiometer Type GK 2021 B combined calomel-glass electrode. The same instrument was adapted for measurements of bromide activity using Orion bromide electrode with Radiometer K401 serving as a reference electrode. In all cases the necessary calibrations were made with standardized solutions.
5. Ultracentrifuge. Sedimentation coefficients characteristic of the native and modified catalases were obtained with Beckman Ultracentrifuge, Model E, using Schlieren Optics.
6. Amino acid analyses. The determination of amino acid composition of catalase preparations were carried out according to the established procedures (55,57) on a Beckman Model 120B amino acid analyzer.

Samples of each catalase preparation were digested in evacuated, sealed tubes by 6 N NaCl at 110° for 20 and 70 hours (four samples at each digestion time). The number of serine and threonine residues were obtained by extrapolation to zero time and the values for valine and isoleucine were



Iron Protoporphyrin IX

Fig. 5

calculated from the 70 hour hydrolysates only. The total half-cystine content of the protein was established by performic acid oxidation of cysteine and cystine to the sulfonic acid stage according to the method of Moore (56).

7. Electrophoresis. Pronase hydrolysates were subjected to electrophoresis by utilizing a water-cooled, flatplate Locarte apparatus; 0.1 M borate buffer, pH 8.2, was used, and a potential of 53 V/cm was applied for 60 min. An amount of hydrolyzate corresponding to 1 to 2 mg catalase was used on each paper strip. Peptides were detected with the cadmium-ninhydrin reagent (60) and by scanning the strips for radioactivity on a Nuclear Chicago Actigraph III.

Assay of ferriprotoporphyrin

The precise evaluation of the prosthetic group content of any hemoprotein is fundamental to subsequent studies of these derivatives. And, since the coenzyme in catalase - as in myoglobin, hemoglobin, plant or mold peroxidases, cytochrome b or tryptophane pyrrolase - is iron protoporphyrin IX (7) (Fig. 5), the assay can be readily accomplished by exploiting its specific absorption characteristics.

Of these, a particularly convenient method relies on the formation of hemochromogens, i.e. derivatives in which iron is in the ferrous state and the fifth and sixth coordination positions are occupied by nitrogenous ligands - characteristically pyridine (64). The 'hemin' containing system is dissolved in 2.48 M pyridine containing 0.07 M

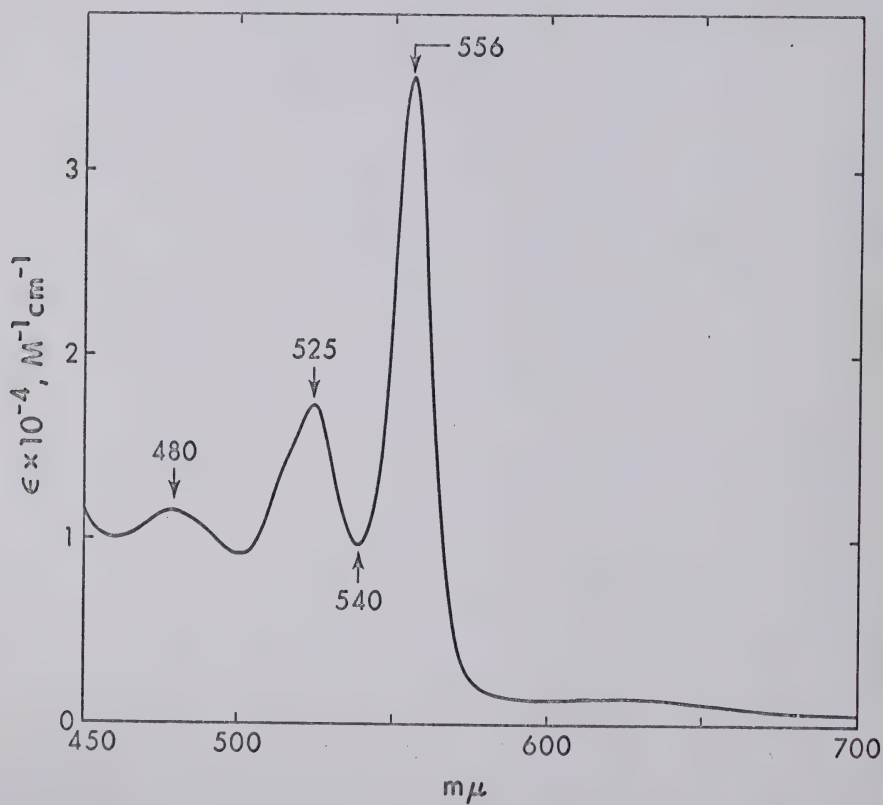


Figure 6. Absorption spectra of the hemochromogen of horse blood catalase
See text for experimental details.

sodium hydroxide and then reduced with a minimum amount of sodium dithionite (65). The spectroscopic features of the resulting derivative, trans-dipyridal ferroprotoporphyrin IX (Fig. 6) provides a sensitive 'fingerprint' of hemin integrity and also permits quantitative evaluations based on (66):

$$\epsilon_{557} = 34.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$$

$$\epsilon_{526} = 17.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$$

$$\epsilon_{540} = 9.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$$

But in spite of the apparent simplicity of this procedure, it is essential to appreciate its limitations (67). Thus, the absorbancies at 557, 540, 526, and 480 mμ must strictly conform to the values obtained with the reference systems such as chromatographically purified ferroprotoporphyrin or crystalline myoglobin. Otherwise, overestimation of hemin may result, particularly if other pigments, ferritin or verdohemes, are present (14). Since the enzyme used in the current studies satisfied the necessary criteria, all the subsequent measurements, whether of catalatic activity or physico-chemical properties of the enzyme, rest on the hemochromogen standard. All catalase concentrations given in this study are in terms of hematin - Fe and not in terms of whole catalase.

Measurement of catalase activity

The catalase mediated decomposition of hydrogen peroxide into oxygen and water - catalatic reaction - is the traditional, although not necessarily the only method of evaluating enzyme activity (68). Three procedures are available for the determination of catalatic activity: manometric, titrimetric or spectrophotometric (69,70).

The first of these relies on the measurement of oxygen pressure whilst the latter rest on the estimation of hydrogen peroxide, either by KMnO_4 titration or by following absorbancy changes in U.V., e.g. at 230 m μ . The simplest methodology is spectrophotometric; it permits the use of low H_2O_2 concentrations ($\epsilon_{\text{H}_2\text{O}_2}^{230} \sim 60$) and allows rapid determination of the pseudo first order (k'') rate constant for the over-all reaction defined by:

$$\frac{d(\text{H}_2\text{O}_2)}{dt} = k'' (\text{H}_2\text{O}_2)$$

and since $k'' = k_1' e = \frac{0.692}{t_{1/2}}$ where e = concentration of ferriprotoporphyrin

(M), $t_{1/2}$ = time for 50% decomposition of hydrogen peroxide and k_1' is the apparent second order rate constant. In turn, k_1' is related to the fundamental rate constants, k_1 and k_4' by (71):

$$k_1' = \frac{2k_1 k_4'}{k_1 + k_4'}$$

where k_1 constitutes the rate constant for compound I formation and k_4' is the constant for compound I - hydrogen peroxide interaction (71):

TABLE VII

Catalatic Activity of Native Catalase

Sample No.	No. of determinations	Final [Cat.] $\times 10^9$, M	$k_1^1 \times 10^{-7}$ $M^{-1} \text{ sec}^{-1}$
1.	6	2.47 to 3.28	0.96
2.	8	1.90	1.07
3.	12	2.20	1.08
4.	42	2.02	1.01
5.	39	2.02	1.03
6.	12	2.39 to 3.19	1.05
7.	3	2.40	1.09
8.	3	2.38	1.28
9.	3	2.92	1.07
10.	12	1.88 to 2.25	1.16
11.	18	1.10 to 4.39	1.06
12.	12	1.54	1.11

5 mM phosphate buffer, pH 7, 25°.

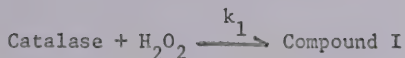
~8 mM H_2O_2 .

TABLE VIII

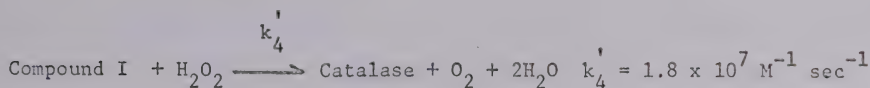
Effect of pH on the Catalatic Activity of Native Catalase

No. of Determinations	Buffer	pH	Final [Cat.] $\times 10^9$, M	$k_1^1 \times 10^{-7}$ $M^{-1} \text{ sec}^{-1}$
4	DMG ¹	3.42	2.39	0.81
6	"	3.98	3.19	0.81
8	"	4.04	2.02	0.76
12	"	4.51	2.39	0.87
9	"	5.48	2.02	0.98
9	"	6.42	2.02	1.00
12	"	7.01	2.02	1.04
27	Phosphate	7.01	2.02	1.03
12	- " - ²	7.11	2.02	1.03
6	- " -	7.98	2.02	1.04
6	Borate	8.00	2.39	0.94
6	- " -	8.98	2.39	0.97
8	- " -	10.01	3.19	0.76

5 mM buffers, 25°, ~8 mM H₂O₂¹ 2,2'-dimethylglutarate.² 1 mM phosphate.



$$k_1 = 6 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$$



Therefore, using horse blood catalase, the expected value for k_1' is:

$$k_1' = \frac{2 \times 6 \times 10^6 \times 1.8 \times 10^7}{6 \times 10^6 + 1.8 \times 10^7} \sim 0.9 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$$

in agreement with the data presented in Table VII.

The successful use of the spectrophotometric method depends, however, on the observance of the following precautions:

- a) the necessary enzyme dilutions must be carried out within the shortest possible time of assay using cold buffers of low ionic strength (e.g. 1 mM phosphate pH 7);
- b) the concentration of catalase should be chosen so that 50 % of peroxide decomposition occurs in 20 - 30 seconds.

This recipe has a firm basis in repeated observations indicating that dilution of the enzyme to the 10^{-9} level (dictated by the extraordinary turnover number of catalase) entails ultimately its inactivation, probably due to the dissociation of the tetrameric enzyme. Similarly, the choice of $t_{1/2} \neq 30$ secs. is imposed by enzyme inactivation - which is also induced by hydrogen peroxide. It is not surprising, therefore, that manometric and titrimetric procedures - which are more time consuming - lead to lower values for the specific activities of catalases; nor is it unexpected that various parameters such as pH (Table VIII), ionic strength, temperature, specific

TABLE IX

Effect of Carboxylic Acids on the Catalatic Activity
of Native Catalase

$$k_1^1 \times 10^{-5}, \text{M}^{-1} \text{sec}^{-1}$$

pH	Phosphate	DMG ¹	Lactate	Acetate	Citrate	Succinate
7.0	103	104	-	-	-	-
6.4	106	100	-	-	103	109
5.5	-	98	-	-	104	86
4.5	-	87	67	-	51	33
4.0	-	81	57	56	23	21
3.4	-	81	46	-	34	19

5 mM buffers, 25°.

1.88 to 3.75×10^{-9} M catalase, ~ 8 mM H_2O_2 .

Each value is the average of at least three determinations.

¹ 2,2'-dimethyl-glutarate.

TABLE X

Effect of Buffer Concentration on the
Catalatic Activity of Native Catalase

Buffer	$k_1^1 \times 10^{-7}, \text{M}^{-1} \text{sec}^{-1}$
1 mM	0.99
5 mM	0.99
10 mM	1.08
50 mM	1.06
0.1 M	0.99
0.2 M	0.97
5 mM (I = 0.11) ¹	1.02

Phosphate buffers, pH 7, 25°.

2.02×10^{-9} M catalase, ~8 mM H_2O_2 .

6 determinations at each buffer concentration.

¹ Containing 0.033 M Na_2SO_4 .

ion effects (Table IX) influence rates of H_2O_2 decomposition. Moreover, the variations in the rates of peroxide decomposition may arise not only by altering the equilibrium between tetrameric enzyme and its inactive subunits, but partly could be contributed by changing the coordination state of the prosthetic group in the presence of hemin ligands such as acetate (72,49).

It is important, however, to appreciate that some of these effects can be overlooked in the assays carried out according to the previously outlined method. For example, in procedures which differ in the manner of enzyme dilution, but are otherwise identical, the resulting activities show substantial variations. Thus, catalase diluted in cold distilled water, or 1 mM phosphate buffer pH 7 ($\mu \sim 2 \times 10^{-3}$), shows essentially the same activity when assayed in 0.001 to 0.2 M phosphates (Tables X); but, if prior to assay reaction the same enzyme is diluted in phosphate buffers of increasing molarity, the activity decreases within 120 secs. to a steady value which depends on the molarity (or μ) of the diluent (73). Therefore, strictly speaking, k'' and hence k_1' , calculated by the half-time method could be undervalued, the error being minimized with decreasing $t_{1/2}$. In view of these reservations, it was essential to show that k'' obtained by the $t_{1/2}$ method is identical to that calculated using integrated first order equation (Eq. 4).

$$k'' = \frac{2.303}{t} \log \frac{(\text{H}_2\text{O}_2)_0}{(\text{H}_2\text{O}_2)_0 - (\text{H}_2\text{O}_2)_t} \quad \text{Eq. 4}$$

This was found to be the case, providing $t_{1/2} < 30$ secs.

Another thorny problem concerns the supposed intrinsic activity of catalases. It has arisen from an intuitively justifiable suspicion that the enzyme prepared in presence of chloroform and ethanol (see p. 19).

may differ from its 'truly native' conformation. The argument is rather teleological since the habitat of catalases in blood corpuscles or liver peroxisomes is not fully defined (74) and attempts to measure enzyme activity in the native state are complicated by diffusion limited transport of peroxide across the membranes. In one case, at least, it was argued that organic solvents cannot be detrimental to enzymic properties since Higashi et al., (75) failed to detect any differences in the catalatic activities of rat erythrocyte hemolysates and of the enzyme purified via the chloroform - ethanol method. It is intriguing therefore that the enzyme obtained without $\text{CHCl}_3/\text{C}_2\text{H}_5\text{OH}$ treatment shows slightly higher overall catalatic activity ($k_1 = 1.5 - 1.8 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$); significantly, its turnover number decreases on storage (2 weeks) or upon crystallization, to the 'typical' value of $k_1' = 0.9 - 1.2 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ (21).

Determination of sulfhydryl groups

The estimation of catalase -SH groups was carried out in 0.33 M acetate, pH 4.6 at 25°. The value of pH chosen for the procedure was dictated by the fact that catalase sulfhydryl groups react faster at pH 4.6 than at pH 7. The reason for this differential reactivity is the high affinity of PCMB for hydroxyl ions, therefore, as the pH is lowered the reactivity of the mercurial is increased (166). Thus, at pH 7 prolonged incubation of the reactants would be required to titrate all the accessible -SH groups; under these conditions other protein functional groups could interact with PCMB (106,107).

The choice of a procedure for the estimation of catalase sulfhydryl groups was also influenced by the fact that the observed absorbance changes are not identical when PCMB forms mercaptides with simple thiol compounds and with protein -SH groups. Such findings have been amply documented (58,166,167) and the observed differences possibly reflect the fact that the chromophore of PCMB finds itself in a different environment after it has reacted with a protein -SH group as compared to the one after its reaction with a simple thiol. Values of $\Delta\epsilon_{255}$, which characterize the mercaptide formation, obtained in this study with catalase illustrate well the statements made above. For the formation of mercaptide with simple thiols the values of $\Delta\epsilon_{255}$ were found to be 6.2×10^3 and 6.9×10^3 $M^{-1} \text{ cm}^{-1}$ (see Table VI). In the case of catalase, values of 10.4×10^3 and 9.7×10^3 $M^{-1} \text{ cm}^{-1}$ were obtained (see calculations on page 35).

To overcome this difficulty, and to make the procedure less time consuming, especially in the case of native catalase, the following procedure was adopted for the estimation of catalase sulfhydryl groups. In a preliminary experiment a rough estimate of the titratable -SH groups of the particular catalase preparation under study was made. Then, to another portion of the same catalase solution a slight excess of PCMB, 20 - 50% over the titratable -SH groups, was added and the reaction was allowed to proceed until no further changes were observed. The unreacted PCMB was back titrated by the addition of small increments of 2-mercaptoethanol (ME) until no further changes were observed upon the addition of the thiol. From the absorbance change observed during the back titration the amount of

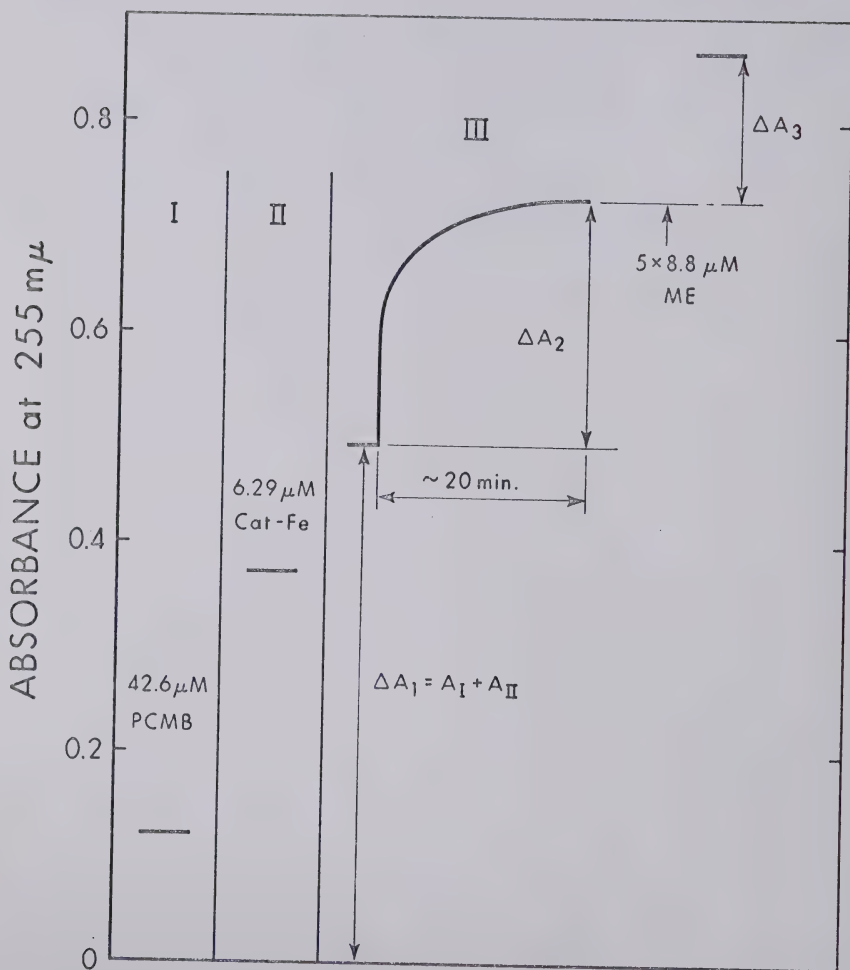


Figure 7. Reaction of PCMB with native catalase.
0.33 M acetate, pH 4.6, 25°.

unreacted PCMB was calculated by using a value of $6.89 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for $\Delta\epsilon_{255}$ (from Table VI). From the initial and unreacted amounts the portion of PCMB that reacted with the protein -SH groups was obtained, and was translated into number of sulphhydryl groups per catalase molecules.

The slight excess of PCMB used was sufficient to make the mercaptide formation relatively fast even in the case of the native enzyme (20 - 30 minutes reaction time); at the same time, the excess PCMB was not great enough to result in the interaction of PCMB with other protein functional groups, and, in addition, the back titration could be carried out rapidly.

The procedure will be illustrated by the description of the titration of native and acid denatured catalases. Furthermore, using acid denatured catalase the more commonly used, "classical", titration procedure will be shown to indicate the reliability of the back titration method adopted for use in these studies (see Figs. 7 and 8).

In Fig. 7 absorbances due to 6.29 μM catalase and 42.6 μM PCMB are shown. After mixing, the interaction of PCMB with catalase -SH groups resulted in a change of absorbance of 0.234, as indicated by ΔA_2 . After no further absorbance change was observed five portions of ME were added, each corresponding to $\sim 8.8 \mu\text{M}$. Although it is not shown in Fig. 7, the last portion of ME did not give rise to a change in absorbance, thus indicating that the back titration of the unreacted PCMB was complete. The total absorbance change was 0.138 as indicated by ΔA_3 . Using a value of $6.89 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (from

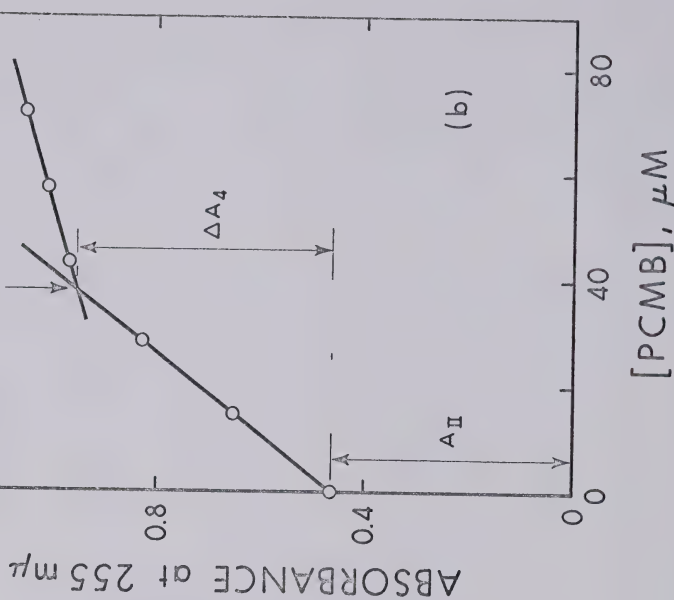
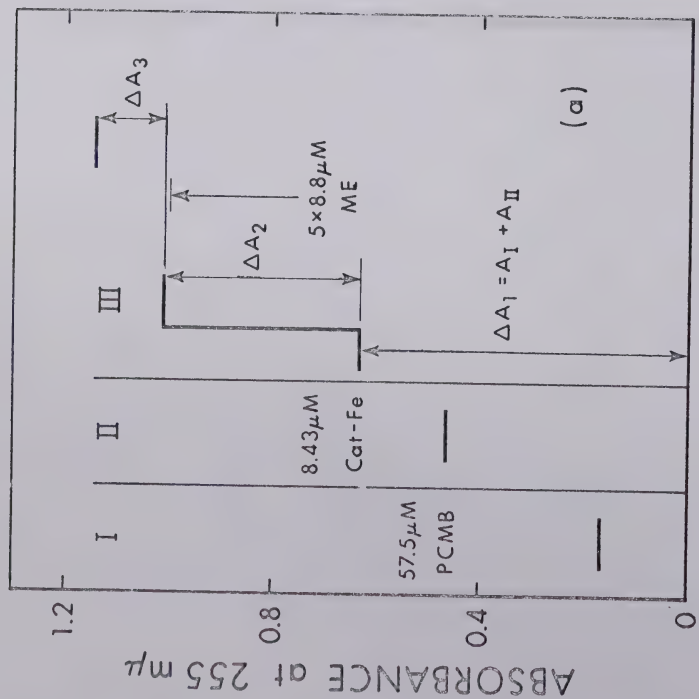


Figure 8. Reaction of acid denatured catalase with PCMB. 0.33 M acetate, pH 4.6, 25°. (a) "Excess PCMB titration method; (b) "Classical" method (see text).

Table VI), the amount of PCMB that was back titrated by ME was found to be 20.0 μM . Therefore, 22.6 μM PCMB had reacted with catalase sulfhydryl groups; from this value the number of -SH groups per catalase molecule was found to be 14.3. In this experiment, ΔA_2 represents the absorbance change associated with mercaptide formation, the value of $\Delta \epsilon_{255}$ calculated from it is 10,400 $\text{M}^{-1} \text{cm}^{-1}$.

In the experiment illustrated by Fig. 8a, the procedure used was the same as in Fig. 7. ΔA_2 corresponded to an absorbance change of 0.368, while ΔA_3 was 0.136. The amount of PCMB that was back titrated with ME was calculated to be 19.7 μM , and therefore 37.8 μM PCMB reacted with catalase sulfhydryl groups. From this value, the number of -SH groups per catalase molecule was calculated to be 17.9. The value of $\Delta \epsilon_{255}$, which accompanies the mercaptide formation was found to be 9,700 $\text{M}^{-1} \text{cm}^{-1}$ in this case.

In Fig. 8b, the same amount of acid denatured catalase, 8.29 μM , was titrated by five additions of PCMB, each corresponding to 14.4 μM . The intersection of the two straight lines defined the endpoint of the titration at 38.0 μM PCMB. That is, this amount of PCMB was required to titrate all the available sulfhydryl groups. Using this value, the number of -SH groups per catalase molecule was found to be 18.0. This figure is almost identical to the result obtained in the experiment illustrated in Fig. 8a, showing that the back titration method, which is more convenient and faster to use in the case of native catalase, is just as reliable as the more commonly used titration procedure.

MODIFICATION OF CATALASE BY BrCN

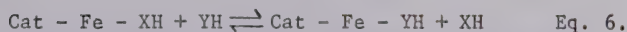
To date, the theories of catalase action generally centered on the reactivity of its prosthetic group and excluded any specific chemical role of the apoenzyme portion of the molecule (22). Within the framework of these hypotheses, the protein solely provides a physical environment modifying the reactivity of hemin - a postulate necessary to accommodate the varied properties of hemoproteins containing the same prosthetic group. An alternate viewpoint, and one which is also more readily tested experimentally, invokes - apart from the specific role of hemin - the direct participation of protein functional group(s) in mediating the reaction characteristics of the system. The idea embodied in this concept is fundamental to most catalyses and has been amply confirmed, not only in model reactions, but also in the investigation of numerous enzymes (131,132,133).

In the case of catalase, the lack of research delineating such neighbouring group participation was not due to the rejection of this approach, but reflected difficulties in formulating a rational line of experimentation. To appreciate more fully the nature of the problem, let us recall that:

- a) the spectrum of catalase remains nearly invariant between pH 4.0 and 10.5, indicating absence of "heme-linked" ionizations which typify the behaviour of other hemoproteins - metmyoglobin, hemoglobin or horse radish peroxidase (22).
- b) in its reaction with hydrogen peroxide, or alkyl hydrogen peroxide, catalase shows no dependence on hydronium ion concentration within the range of pH 4 - 11. The sharp decrease in activity outside these limits is most likely due to an irreversible protein denaturation (22,112).

- c) the exchange of ligands, assumed to occur at the sixth coordination position of the prosthetic group, is also pH independent, providing the rate expression is formulated according to (22):

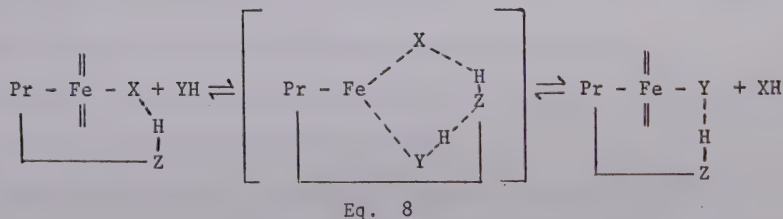
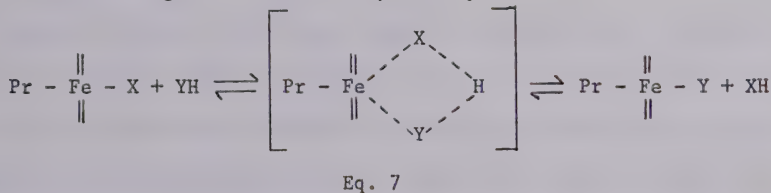
$$v = k (\text{cat} - \text{Fe} - \text{XH}) (\text{YH}) \quad \text{Eq. 5.}$$



where $\text{YH} = \text{HF}, \text{HCO}_2\text{H}, \text{CH}_3\text{CO}_2\text{H}, \text{HCN}, \text{HN}_3$,

and XH is generally identified with H_2O , or OH , and is the group displaced in the course of reaction.

However, in spite of its apparent simplicity (Eq. 6), the ligand interchange reaction does not necessarily proceed by a direct proton transfer between the leaving and entering groups (Eq. 7), but according to Nicholls and Schonbaum (22) could involve participation of general acid - general base catalysis (Eq. 8).



This idea is supported by observation of catalase inhibition following incorporation of 3-amino-1,2,4-triazole into the protein matrix (93) and by the stereospecific oxidation of ethanol by catalase compound I (134) which demands specific pathways for solvation of

liberated protons. Regarding ZH (Eq. 8), the most likely candidate had to be a group of $pK_a \geq 12$ (arginine ?) since its involvement would reconcile the observed pH invariance of catalytic behaviour and would fulfil the predicted general acid - general base function (22).

Therefore, if this hypothesis had any substance, the specific modification of such a group should be possible. Ideally, the necessary reagent had to have sufficient stability and solubility in aqueous systems; be subject to nucleophilic substitution; possess the capacity to interact with metal ions and/or be capable of forming charge transfer complexes. Considering these requirements, Schonbaum investigated the interaction of catalase with electrophilic reagents and was led to the discovery of specific enzyme modifications by the pseudo halogen-cyanogen bromide.

Cyanogen bromide is not a new reagent in biochemistry. Introduced by Gross and Witkop (135) as an application of the classical von Braun reaction, it is mistakenly believed to show unique specificity for the cleavage of methionine peptide bonds. Undoubtedly, in this respect it is very effective, but the reaction generally requires a high ratio of reagent to protein and is only specific providing the pH is sufficiently low to prevent reaction with other potential nucleophiles- α - or ϵ -amino, imidazole, sulfhydryl groups.

Before describing the experimental results some information will be given here about various catalase derivatives; the experimental evidence supporting these statements is presented in Chapter IV. When native catalase, cyan-catalase and pretreated

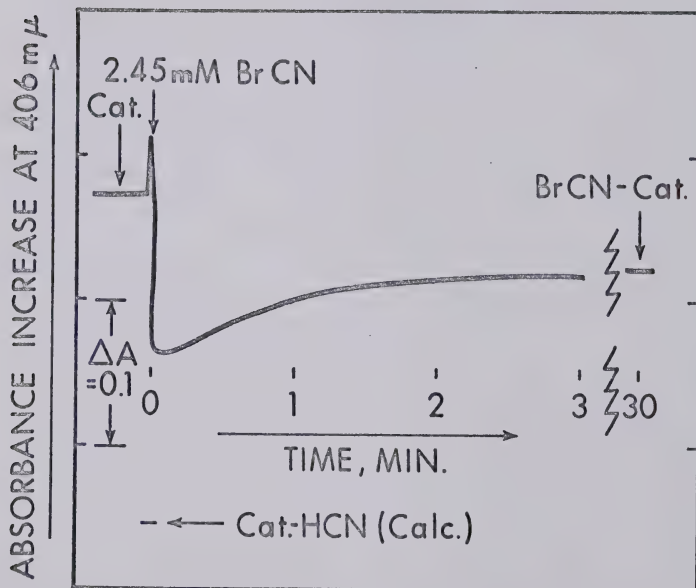


Figure 9. Reaction of BrCN with native catalase.
10 mM phosphate, pH 7.2, 25°; 3.7 μ M catalase.

catalase* react with excess BrCN, the same catalase derivative is formed as shown by their spectroscopic properties. This BrCN-modified derivative is enzymatically inactive (95 to 99% inhibition). On the other hand pretreatment, and for that matter PCMB-treatment, does not effect the catalytic efficiency of the enzyme.

Reaction of cyanogen bromide with native catalase

As in its reaction with beef liver or porcine blood catalases (49), cyanogen bromide interacts with horse blood catalase in a two-stage process (Fig. 9). The first, or fast reaction stage-appeared to be attended by the release of cyanide ions and does not lead to enzyme inhibition; the second, or "slow" phase of the reaction, gives a stable and inactive enzyme derivative.

The first stage of the reaction, represented by the rapid decrease in absorbance in Fig. 9, is the reaction of BrCN with catalase sulphydryl groups, as will be shown later (see page 40), and as the result cyanide is released which forms a complex with the enzyme. The second phase of the reaction, the slow increase in absorbance in Fig. 9, is the inhibition of catalase by BrCN which is present in sufficient excess. The increase in absorption results from the conversion of cyan-catalase to BrCN-modified catalase. To define the inhibition reaction more closely, attempts were made to elucidate the nature of the initial reaction so that the effects of the liberated cyanide would be eliminated.

* Pretreated catalase is prepared by oxidizing the -SH groups of the enzyme with BrCN in the presence of 0.3 M formate (see page 46).

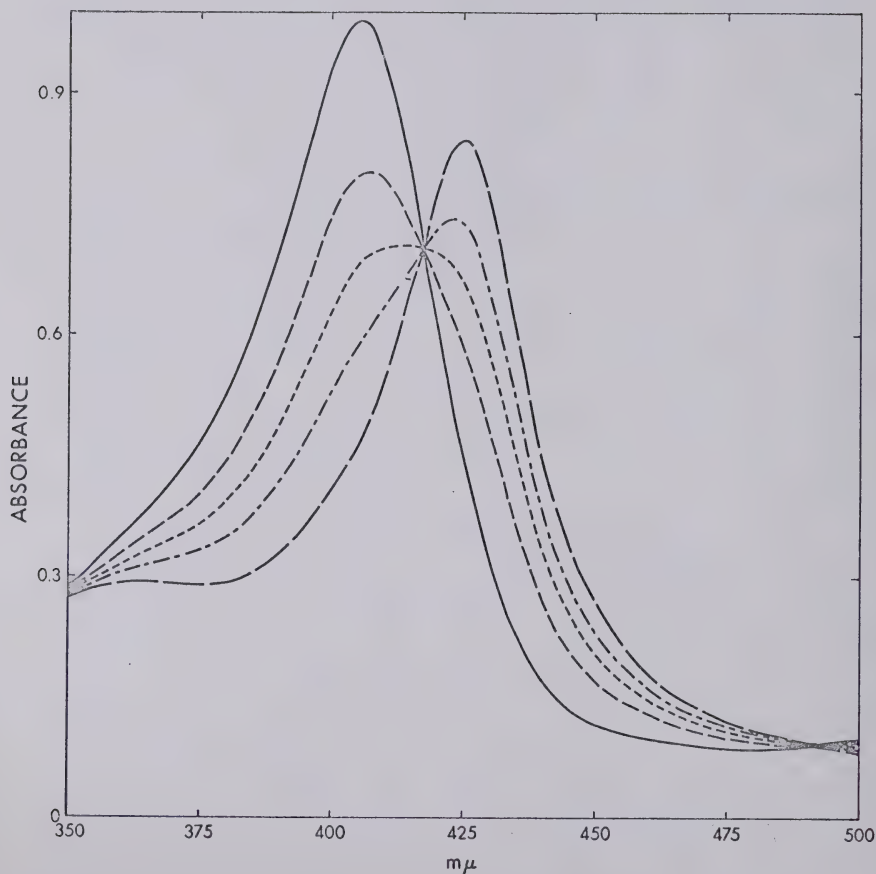


Figure 10. Reduction of cyanogen bromide by native catalase. Addition of KCN to the native enzyme. 0.1 M phosphate, pH 7.0, 25°; 8.5 μ M catalase. Curve (—): no KCN; Curve (---): 5.2 μ M KCN; Curve (----): 10.4 μ M KCN; Curve (— · — · —): 20.6 μ M KCN; Curve (— — —): ~28 mM KCN.

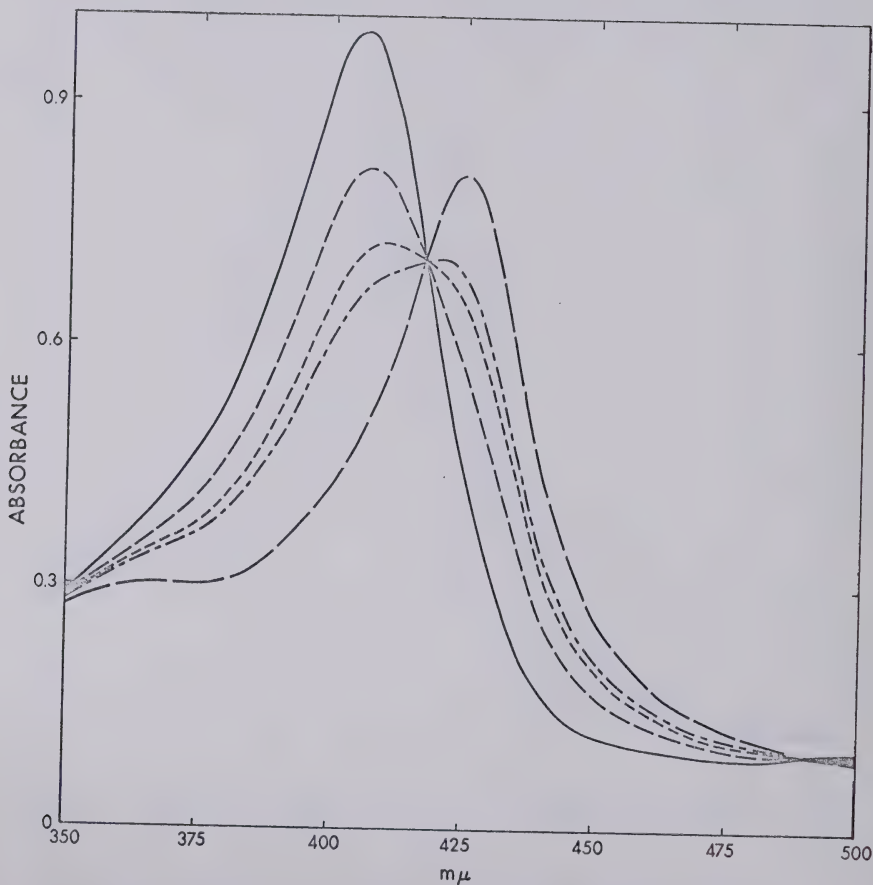


Figure 11. Reduction of cyanogen bromide by native catalase.
 Addition of BrCN to the native enzyme.
 0.1 M phosphate, pH 7.0, 25°; 8.5 μ M catalase.
 Curve (—): no BrCN; Curve (---): 5.2 μ M BrCN;
 Curve (-·-·-): 10.4 μ M BrCN; (Curve (— — — — —): 15.5 μ M BrCN;
 Curve (— — — — —): \sim 0.9 mM KCN.

TABLE XI

Titration of Native Catalase with BrCN

[BrCN] _{Total}	A ₄₀₅	A ₄₂₅	ΔA_{405}	ΔA_{425}	[Fe-HCN] ^a	[Fe _{free}]	[HCN _{free}] ^b	Total [HCN] ^c
					μM	μM	μM	μM
-	.985	.445	-	-	-	-	-	-
5.2 μM BrCN	.814	.572	.171	.127	2.74	5.74	2.50	5.2
10.4 μM BrCN	.708	.651	.277	.206	4.46	4.06	5.77	10.3
15.5 μM BrCN	.655	.685	.330	.240	5.32	3.18	8.79	14.1
0.9 μM KCN	.475	.809	.510	.364	8.10	-	-	-

8.5 μM catalase, 0.1 M phosphate, pH 7, 25°.

^a Calculated from ΔA values by using $\Delta \epsilon_{405} = 6.2 \times 10^4 M^{-1} cm^{-1}$, and
 $\Delta \epsilon_{425} = 4.6 \times 10^4 M^{-1} cm^{-1}$.

^b Calculated by using $5.25 \times 10^{-6} M$ as the dissociation constant of catalase-HCN.

^c Sum of [Fe-HCN] and [HCN_{free}].

Reduction of cyanogen bromide

The aim of these experiments was to identify cyanide as the product of the initial reaction between BrCN and native catalase (see Fig. 9). In the control experiment small amounts of KCN were added to catalase to obtain a series of spectra shown in Fig. 10. In the other experiment, similar amounts of BrCN were added to an identical catalase solution; spectra were taken after sufficient time was allowed for the completion of the reaction, 5 - 10 minutes (Fig. 11). The similarity of the two sets of spectra indicate that cyanide is liberated in the initial reaction between catalase and BrCN. It is apparent that the inhibition by BrCN is much slower than the liberation of cyanide, which is the result of a reaction between BrCN and protein functional groups, because the small amounts of BrCN added is quantitatively converted to cyanide (see Table XI). Possible reservations that the reaction represents artifacts, arising from cyanide contamination, or BrCN-hydrolysis, were eliminated in companion experiments - using horse radish peroxidase, an enzyme which readily combines with cyanide ($K_{\text{diss}} = 2 \times 10^{-6} \text{ M}$) but, unlike catalase, does not react with BrCN.

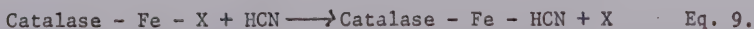
Reaction of native catalase with BrCN in the presence of added cyanide

In order to characterize the second stage of the reaction when BrCN is added to native catalase the possible effect of the cyanide liberated in the first step had to be eliminated. The simplest solution appeared to be the addition of large amounts of cyanide, 100 to 200 μM , to catalase before the BrCN treatment. In this manner the

cyanide liberated in the first step would not change the conditions significantly and thus the second stage of the reaction could be monitored; the rate constant obtained in this manner could be corrected for the presence of the known amount of cyanide. The mathematical solution of this problem was obtained by assuming that KCN and BrCN are competing for the same reaction site of the enzyme, and also that the BrCN - modified enzyme does not bind cyanide under the conditions used ($[KCN] \neq 50 [Cat \cdot Fe]$).

However, for the application of this method the value of the dissociation constant of cyan-catalase, K_{diss} , was required over the entire pH range of 4 to 10, which was to be investigated to observe the effect of pH on catalase - BrCN reaction. Therefore, the determination of the dissociation constant of cyan-catalase will be given before the results of the cyan-catalase-BrCN reaction are described.

Determination of the dissociation constant of cyan-catalase. The interaction of catalase with cyanide is an example of the general reactivity of hemoproteins containing a labile ligand at the sixth coordination position (22). The dissociation constant represents the reaction outlined in Eq. 9, since an alternate expression, in terms of cyanide ion fails to account for the involvement of hydrocyanic acid in the rate limiting step of the ligand interchange (13).



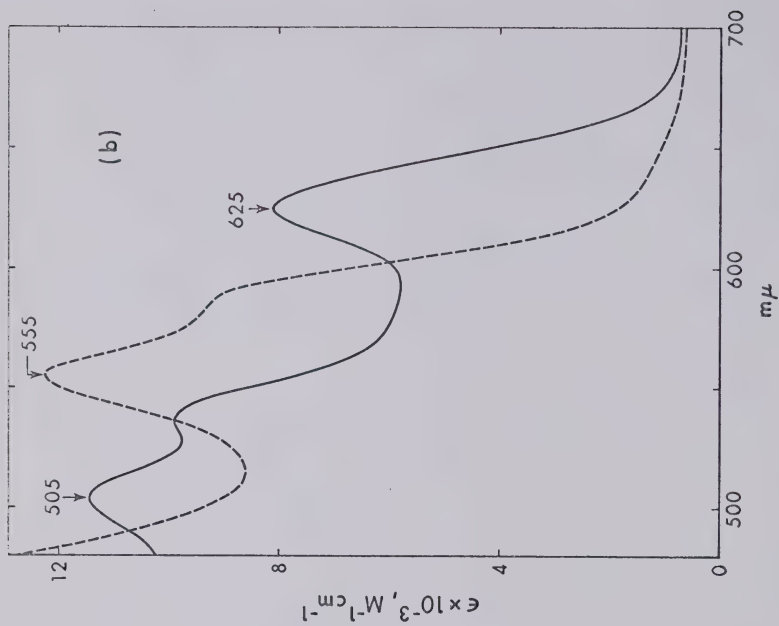
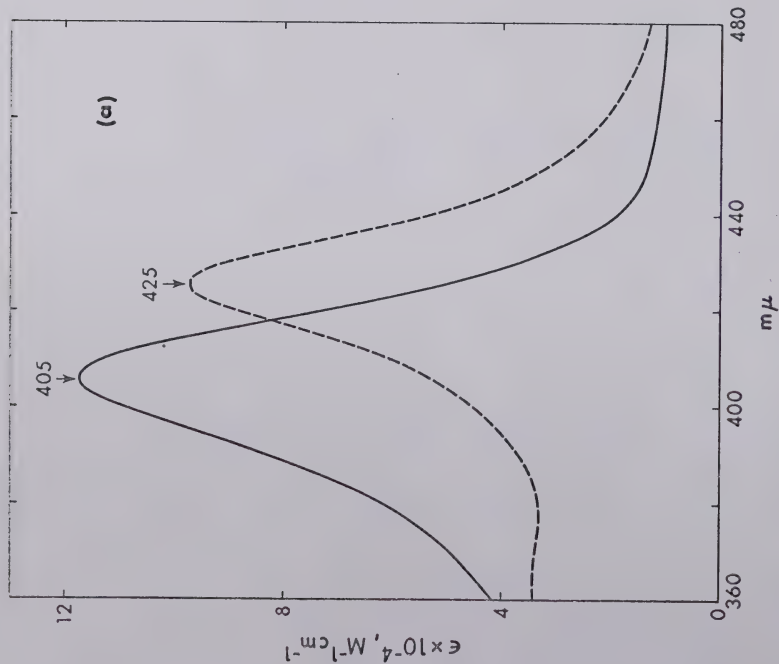


Figure 12. Absorption spectra of catalase and its cyanide derivative in the Soret (a) and visible (b) regions. 0.1 M phosphate, pH 7.0, 25°. Catalase (—), catalase-HCN (---).

Chance had investigated the effect of pH on the dissociation constant of cyan catalase (13) and found that it was pH independent in the pH region of 4 - 7, but observed an unexplainable increase in the pH range of 7 to 10 (see Table XIII). Therefore, it was felt that the determination of the K_{diss} in the high pH range was necessary to obtain meaningful results in the cyan-catalase-BrCN experiments.

The procedure takes advantage of the spectroscopic changes which attend the conversion of the native enzyme into its cyanide derivative. Figure 12 shows the absorption spectra of catalase and its cyanide derivative, which was formed in the presence of about 500 μ M HCN. From the observed absorbance changes resulting upon the addition of known, non-saturating amounts of HCN, and the previously evaluated differences between molar extinction coefficients the amount of complex (Fe-HCN) can be calculated. These values can be used in a graphical procedure, which is described by Eq. 10.

$$\frac{1}{[\text{Fe} \cdot \text{HCN}]} = \frac{1}{[\text{Fe}]_T} + \frac{K_{diss} (K_a + [\text{H}^+])}{[\text{Fe}]_T [\text{H}^+]} \times \frac{1}{[\text{CN}]_T - [\text{Fe} \cdot \text{HCN}]} \quad \text{Eq. 10.}$$

This formulation follows upon solution of conservation relationships:

$$[\text{Fe}]_T = [\text{Fe}]_f + [\text{Fe} - \text{HCN}]$$

$$\text{and} \quad [\text{CN}]_T = [\text{CN}^-] + [\text{HCN}] + [\text{Fe} - \text{HCN}]$$

$$\text{where} \quad [\text{Fe}]_T = \text{total hematin-iron}$$

$$[\text{Fe}]_f = \text{concentration of hematin-Fe not liganded to cyanide}$$

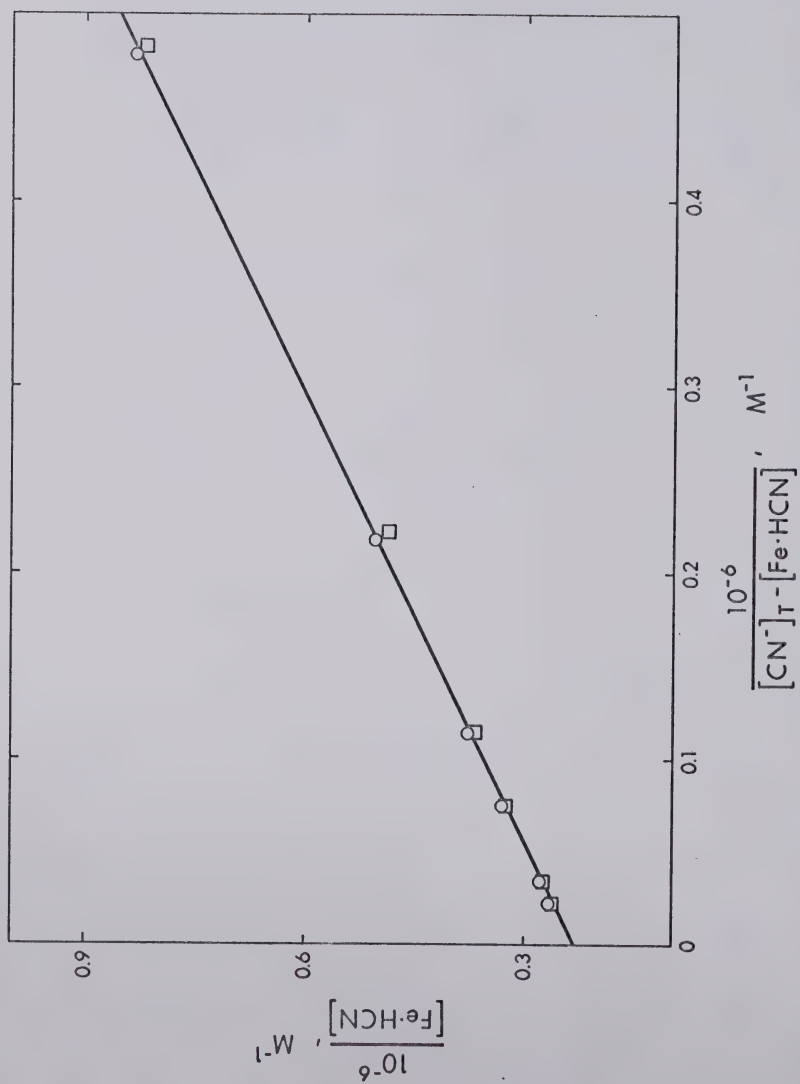


Figure 13. Determination of the dissociation constant of catalase-cyanide complex. Data from Table XII.

TABLE XII

Determination of the Dissociation Constant of Catalase-HCN

At 405 mμ

[CN] _T	ΔA	[Fe·HCN], μM	$\frac{10^{-6}}{[\text{Fe} \cdot \text{HCN}]}$	[CN] _T - [Fe·HCN] μM	$\frac{10^{-6}}{[\text{CN}]_T - [\text{Fe} \cdot \text{HCN}]}$
3.29 μM	0.076	1.22	0.820	2.07	0.483
6.58 μM	0.129	2.06	0.486	4.52	0.222
11.45 μM	0.170	2.72	0.386	8.73	0.115
16.35 μM	0.192	3.07	0.326	13.28	0.075
32.60 μM	0.227	3.64	0.275	28.96	0.035
48.90 μM	0.240	3.84	0.260	45.06	0.022

At 430 mμ

3.29 μM	0.068	1.20	0.834	2.09	0.478
6.58 μM	0.112	1.98	0.505	4.60	0.218
11.45 μM	0.150	2.64	0.379	8.81	0.114
16.35 μM	0.171	3.02	0.331	13.33	0.075
32.60 μM	0.203	3.58	0.280	29.02	0.034
48.90 μM	0.213	3.76	0.266	45.14	0.022

$$\Delta\epsilon_{405} = 6.25 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1},$$

$$\Delta\epsilon_{430} = 5.67 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}.$$

0.1 M phosphate, pH 6.98, 25°, 4.32 μM catalase.

$$K_{\text{diss}} = \text{Slope} \cdot \frac{[\text{Fe}]_T \cdot [\text{H}^+]}{K_a + [\text{H}^+]} = \text{Slope} \cdot \frac{4.32 \times 10^{-6} \times 1.05 \times 10^{-7}}{7.2 \times 10^{-10} + 1.05 \times 10^{-7}} =$$

$$= \text{Slope} \times 4.28 \times 10^{-6}$$

(Cont'd.)

TABLE XII (Cont'd.)

Determination of the Dissociation Constant of Catalase-HCN

From Fig. 11: y intercept = 0.230×10^6

$$\therefore [\text{Fe}]_T = \frac{1}{0.230 \times 10^6} = 4.34 \times 10^{-6} \text{ M}$$

Slope = 1.24

$$\therefore K_{\text{diss}} = 1.24 \times 4.28 \times 10^{-6} = 5.30 \times 10^{-6} \text{ M.}$$

TABLE XIII

Dissociation Constant of Catalase-HCN

pH	7.0	8.1	9.0
	5.08	4.95	
$K_{\text{diss}} \times 10^6, \text{ M}$	5.16	4.84	5.25
	5.30	4.94	5.41
	5.48	4.91	
Average: $K_{\text{diss}} \times 10^6, \text{ M}$	5.25	4.91	5.33
pK	5.28	5.31	5.28

0.02 M and 0.1 M phosphate buffers, pH 7.0 and 8.1, 0.05 M borate buffers, pH 8.1 and 9.0, 25°.

4.2 to 4.3 μM catalase

Effect of pH on the pK_{diss} of Catalase-HCN

pH	7.0	7.2	8.0	9.0	10.0
pK_{diss} , acc. to Chance ^a	-	5.04	4.89	4.47	4.21
pK_{diss} , this work	5.28	-	-	5.28	-

^a From Fig. 5 of Ref. 13. The value of $\log \left(\frac{K_a}{[\text{H}^+]} + 1 \right)$ was added to obtain figures listed here.

TABLE XIV

Reaction of Native Catalase with BrCN in Presence of Added KCN

[KCN], μM	[BrCN], mM	$\frac{10^{-2}}{[\text{BrCN}]}, \text{M}^{-1}$	$k_{\text{obs}} \times 10^4, \text{sec.}^{-1}$				
			pH 4	pH 5	pH 6	pH 7	Average
187	0.468	21.4	10.1	12.3	10.0	11.0	10.8
"	0.935	10.7	20.0	21.2	21.7	23.0	21.5
"	1.40	7.15	32.0	34.0	32.2	34.4	33.2
"	1.87	5.35	34.2	44.5	38.7	47.0	41.2
93.5	0.140	71.5	6.70	6.87	7.14	6.50	6.80
"	0.468	21.4	18.7	20.0	20.0	22.5	20.4
"	0.935	10.7	37.5	38.4	36.3	42.3	38.6
"	1.40	7.15	58.5	61.7	45.2	64.5	57.5

0.1 M buffers, pivalate of pH 4 and 5, phosphate at pH 6 and 7,
25°.

4.5 to 4.7 μM catalase.

The average values of k_{obs} were used in Fig. 16.

From Fig. 16:

1. Slope = $4.17 \times 10^{-1} \text{ M sec}$ (line marked with \square 's).

$$k = \frac{[\text{CN}]_{\text{T}}}{k_{\text{diss}}} \times \frac{1}{\text{slope}} = \frac{187 \times 10^{-6}}{5.25 \times 10^{-6}} \times \frac{1}{0.417} = 85.3 \text{ M}^{-1} \text{ sec}^{-1}$$

2. Slope = $2.20 \times 10^{-1} \text{ M sec}$ (line marked with O's).

$$k = \frac{[\text{CN}]_{\text{T}}}{k_{\text{diss}}} \times \frac{1}{\text{slope}} = \frac{93.5 \times 10^{-6}}{5.25 \times 10^{-6}} \times \frac{1}{0.220} = 81.0 \text{ M}^{-1} \text{ sec}^{-1}$$

$[\text{CN}]_T$ = cyanide + hydrocyanic acid

K_{diss} = dissociation constant defined in Eq. 9.

K_a = dissociation constant of HCN

Plotting then $\frac{1}{[\text{Fe} - \text{HCN}]}$ vs. $\frac{1}{[\text{CN}]_T - [\text{Fe} - \text{HCN}]}$

permits calculation of K_{diss} from the slope of the resulting straight line. An example of such a calculation is presented in Table XII and Fig. 13. Extending the measurements over pH 7 - 9 gave essentially invariant K_{diss} in agreement with Chance's findings at pH 7, but contrasting to a small increase in K_{diss} he noted above pH 7 (Table XIII). The most facile explanation for the observed divergence is the known instability of catalase with increasing pH; it can be only assumed that different preparations might show variations in this respect. An alternate explanation invoking ionization of an enzyme functional group, for example by analogy to behaviour of myoglobin, seems less likely since no pH dependence was established in reactions of catalase with other potential ligands.

Reaction of cyan-catalase with BrCN. The conditions, under which the experiments were carried out, are given in Table XIV. BrCN was added to stoppered cuvettes containing the indicated amounts of catalase and KCN, and then the formation of the BrCN-modified catalase was followed spectrophotometrically at 425 m μ . The values of k_{obs} were calculated from the spectrophotometric traces by using the graphical

first order method (see Appendix). Each value of k_{obs} given in Table XIV at a particular pH value and BrCN concentration, is the average of two determinations.

Keeping in mind the assumptions outlined at the beginning of this section, the rate of modification of catalase by BrCN can be described by the following equation:

$$v = \frac{d(\text{modified enzyme})}{dt} = \frac{k[\text{BrCN}]}{1 + \frac{[\text{CN}]_T}{K_{\text{diss}}}} \times ([\text{Fe}]_T - [\text{Fe}'])$$

$$= k_{\text{obs}} ([\text{Fe}]_T - [\text{Fe}'])$$

where k is the true second order rate constant for the reaction between catalase and BrCN; $[\text{CN}]_T$ and $[\text{Fe}]_T$ have been defined before (see page 42) and $[\text{Fe}']$ represents the concentration of the BrCN-modified enzyme.

One additional condition has to be imposed to insure the application of the above equation: the concentrations of both HCN and BrCN should be fifteen to twenty-fold higher than the concentration of catalase hematin-Fe.

From the above equation we get:

$$k_{\text{obs}} = \frac{k[\text{BrCN}]}{1 + \frac{[\text{CN}]_T}{K_{\text{diss}}}}$$

The rearrangement of this equation leads to the following expression:

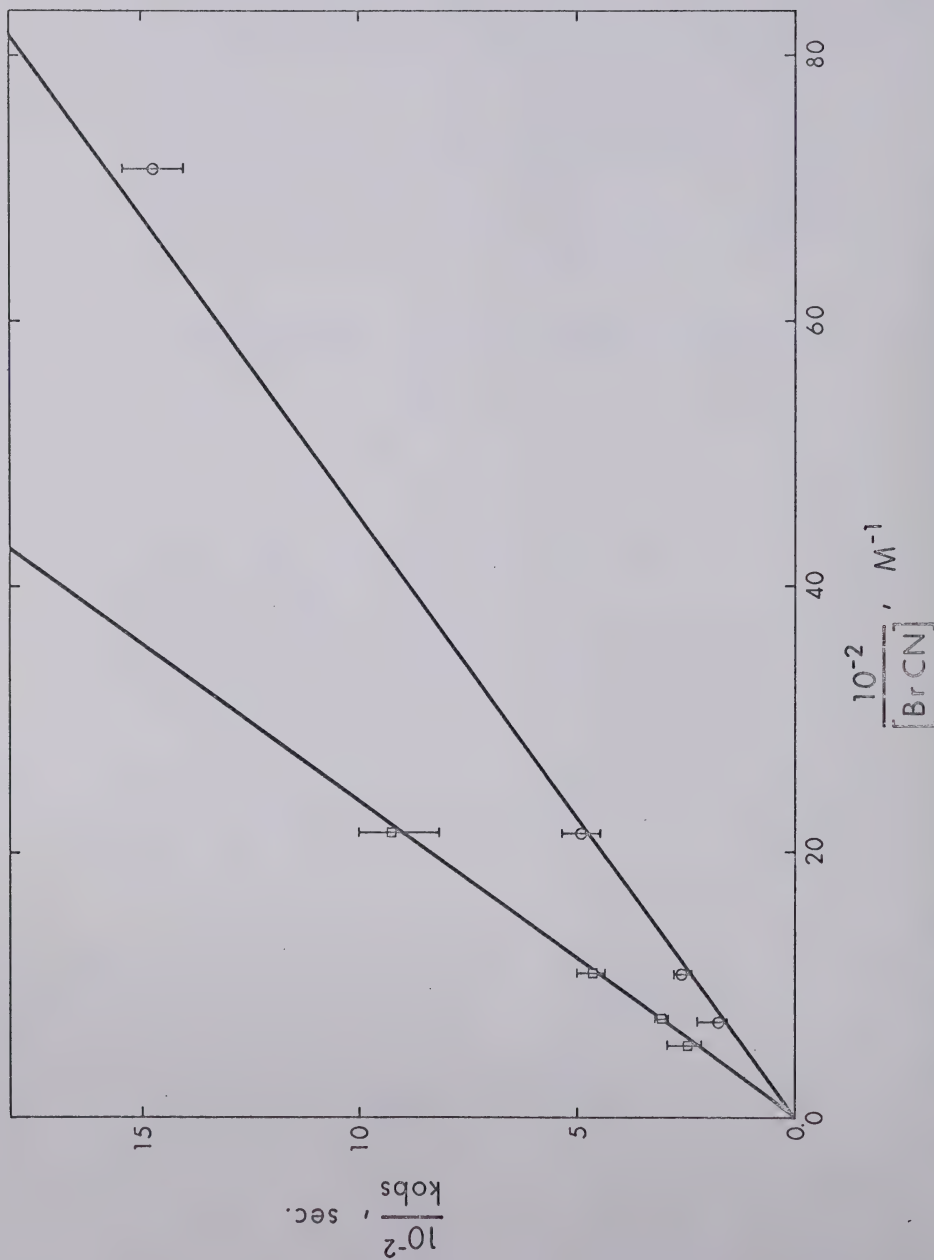


Figure 14. Reaction of cyan-catalase with BrCN. Graphical determination of the second order rate constant k (see also Table XIV).

$$\frac{1}{k_{\text{obs}}} = \frac{1}{k[\text{BrCN}]} + [\text{CN}]_T \cdot \frac{1}{k \cdot K_{\text{diss}} \cdot [\text{BrCN}]}$$

$$\frac{1}{k_{\text{obs}}} = \frac{1}{k[\text{BrCN}]} \left(\frac{K_{\text{diss}} + [\text{CN}]_T}{K_{\text{diss}}} \right)$$

When $[\text{CN}]_T \gg K_{\text{diss}}$, then the above equation becomes:

$$\frac{1}{k_{\text{obs}}} = \frac{1}{k[\text{BrCN}]} \cdot \frac{[\text{CN}]_T}{K_{\text{diss}}}$$

and therefore by plotting $1/[\text{BrCN}]$ against $1/k_{\text{obs}}$ a straight line is obtained. From the slope of the line the intrinsic value of k can be calculated.

The use of graphical method and calculation of k from the data is illustrated in Table XIV and Fig. 14.

The k_{obs} values listed in Table XIV indicate that pH does not have an effect on the reaction rate in the pH range examined. Although there are significant differences observed, there is no definite tendency evident; the observed variations are probably due to technical shortcomings.

The rate measurements were not carried out at pH values higher than 7 because the method was found to be unsuitable to give reliable results in alkaline solutions. The increasing amounts of cyanide ion at the higher pH values appeared to complicate the kinetic analysis, and furthermore, native catalase and especially its BrCN-modified derivative became progressively more unstable with increasing pH (see page 48). This instability of the enzyme and its BrCN-modified derivative made rate measurements impossible. The

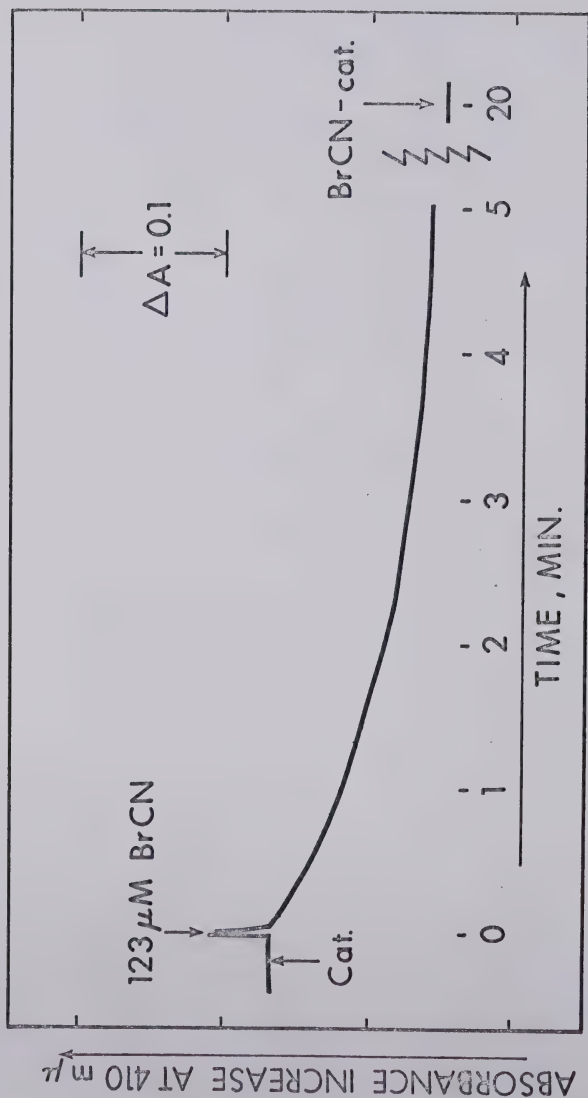


Figure 15. Reaction of PCMB - treated catalase with BrCN. 0.1 M phosphate, pH 7.0, 25°; 6.5 μM catalase.

determination of the rate constant at three higher pH values was accomplished under more favorable conditions; these results are presented in another section of this Chapter (see page 49).

Oxidation of sulfhydryl groups

The difficulties described in the previous section indicated that a better method had to be found to eliminate the liberation of cyanide when catalase is treated with BrCN. Since it was thought that the cyanide liberation was due to the reaction of catalase sulfhydryl groups, it was obvious that the blocking of -SH groups would solve the problem easily.

Justification of the above approach came from the following experiment. Catalase was treated with excess PCMB ($[\text{PCMB}]/[\text{cat}\cdot\text{Fe}] = \sim 10$) at pH 7 and 25° for 8 hours, then the excess PCMB was dialyzed off. When BrCN was added to a sample of this PCMB - treated catalase only a single phase reaction was observed (see Fig. 15). There was no evidence that the inhibition reaction was preceded by the liberation of cyanide, confirming that the cyanide was the product of the reaction between the sulfhydryl groups and BrCN in the case when untreated, native catalase was used.

The experiment illustrated in Fig. 15 also indicates that cyanide protects against BrCN-modification, since the previously observed rate of inhibition ($k_{\text{app}} \sim 15 \text{ M}^{-1} \text{ sec}^{-1}$) obtained with untreated catalase increased substantially here ($k_{\text{app}} \sim 63 \text{ M}^{-1} \text{ sec}^{-1}$). This finding justifies the assumption made in the last section that cyanide and BrCN competes for the same site on the enzyme.

In the long run, however, it appeared that PCMB was not entirely satisfactory for the blocking of -SH groups, mainly because the possibility existed that free sulfhydryl groups could be liberated in PCMB-treated catalase if the excess PCMB is dialyzed off. Faced with this dilemma it was decided to oxidize the sulfhydryl groups with BrCN while the enzyme was protected against the specific inhibition by a ligand at the sixth coordination position of the heme group.

Formate was chosen as the protecting agent mainly because of its high affinity for catalase-hematin, ($K_{diss} \sim 6 \times 10^{-6}$ M), which is equalled only by azide and cyanide. However, unlike azide, formate does not react with BrCN, and in contrast to cyanide it is far easier to handle. The optimum amount of ligand was determined by using formate concentrations ranging from 0.1 to 0.5 M; at 0.1 M formate, pH 4.5, substantial inhibition of the enzyme still occurred, while at 0.5 M the enzyme was no longer entirely soluble. After various trials, the desired conditions were obtained in 0.3 M formate, pH 4.5 ± 0.1 , using approximately ten to twenty-fold excess of BrCN with respect to catalase hematin. After four to eight hours of incubation, the product was dialyzed, first against 0.3 M formate - to remove the excess BrCN - and then against 10 mM phosphate, pH 7. Catalase obtained in this manner - which will be referred to as "pre-treated enzyme" - retained only vestigial amounts ($2 \pm 2\%$) of its cyanide liberating capacity when treated with BrCN; it also generally contained less than 5% of the modified enzyme.

TABLE XV

Reaction of Pretreated Catalase with BrCN

Exp. No.	Monitored at $\mu\mu$	Reaction order	[Catalase], μM	[BrCN], μM	k $\text{M}^{-1}\text{sec.}^{-1}$
1	417.8	2nd	5.30	6.56	160
2	620	"	51.0	87.0	162
3	410	1st	6.60	65.5	122
4	"	"	6.16	107	152
5	"	"	6.16	107	138
6	"	"	6.18	107	137
7	"	"	6.20	111	114
8	"	"	6.23	111	148
9	"	"	6.18	111	137

0.05 M to 0.1 M phosphate buffers, pH 7.0, 25°.

Reaction of pretreated catalase with BrCN

It has been shown previously (see Table XIV) that, in the presence of added cyanide, the second order rate constant of catalase modification was independent of hydronium ion concentration in the pH range of 4 to 7. Having obtained the pretreated enzyme, the catalase-BrCN reaction could now be examined directly and the pH dependence of the reaction examined on the alkaline side of neutrality.

Reaction at pH 7. The reaction of pretreated catalase with BrCN was examined under the conditions given in Table XV. The values of the second order rate constants were obtained from the spectrophotometric traces by using the appropriate graphical method under first or second order conditions (see Appendix). The average of the values given in Table XV is $140 \text{ M}^{-1} \text{ sec}^{-1}$. This figure is at least 50% higher than that obtained in the presence of added cyanide. This substantial variation appears to question the reliability of the previous method, and probably expresses a difference in the conformational states of catalase and its cyanide derivative. The merits of this hypothesis would have been readily tested, but the experiments were deferred in a continuing endeavour to establish an unequivocal relationship between the inhibition rate constant and hydronium ion concentration.

Reaction at pH 7 to 10. With the pretreated enzyme, the 'side effects' of cyanide are eliminated and, in principle, it should have been possible to carry out kinetic evaluation to the usual limits of protein (and BrCN) stability, i.e. to $\text{pH } 10.5 \pm .5$. Hopes of achieving this

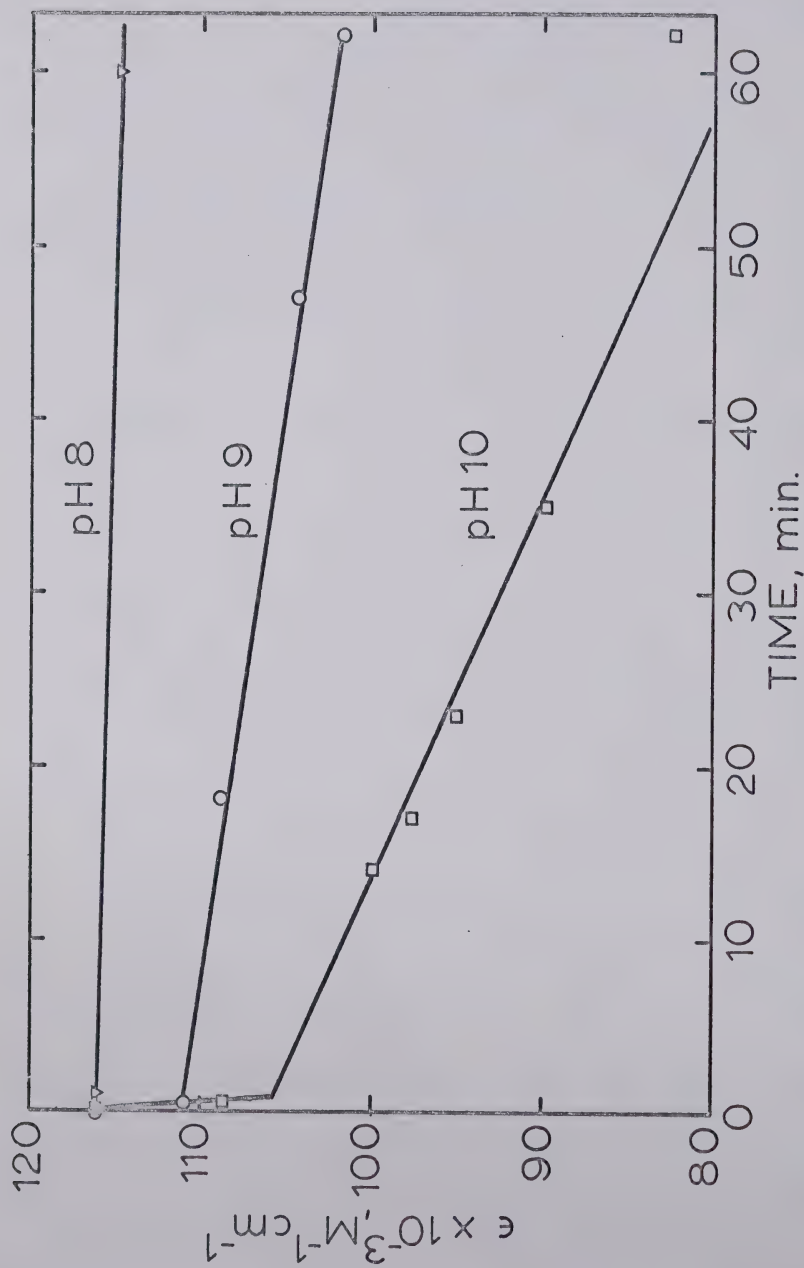


Figure 16. Effect of pH on the absorbance of pretreated catalase in the Soret region. Apparent ϵ values at 405 m μ . 0.1 M borate buffers, 25°, 5.3 μ M catalase.

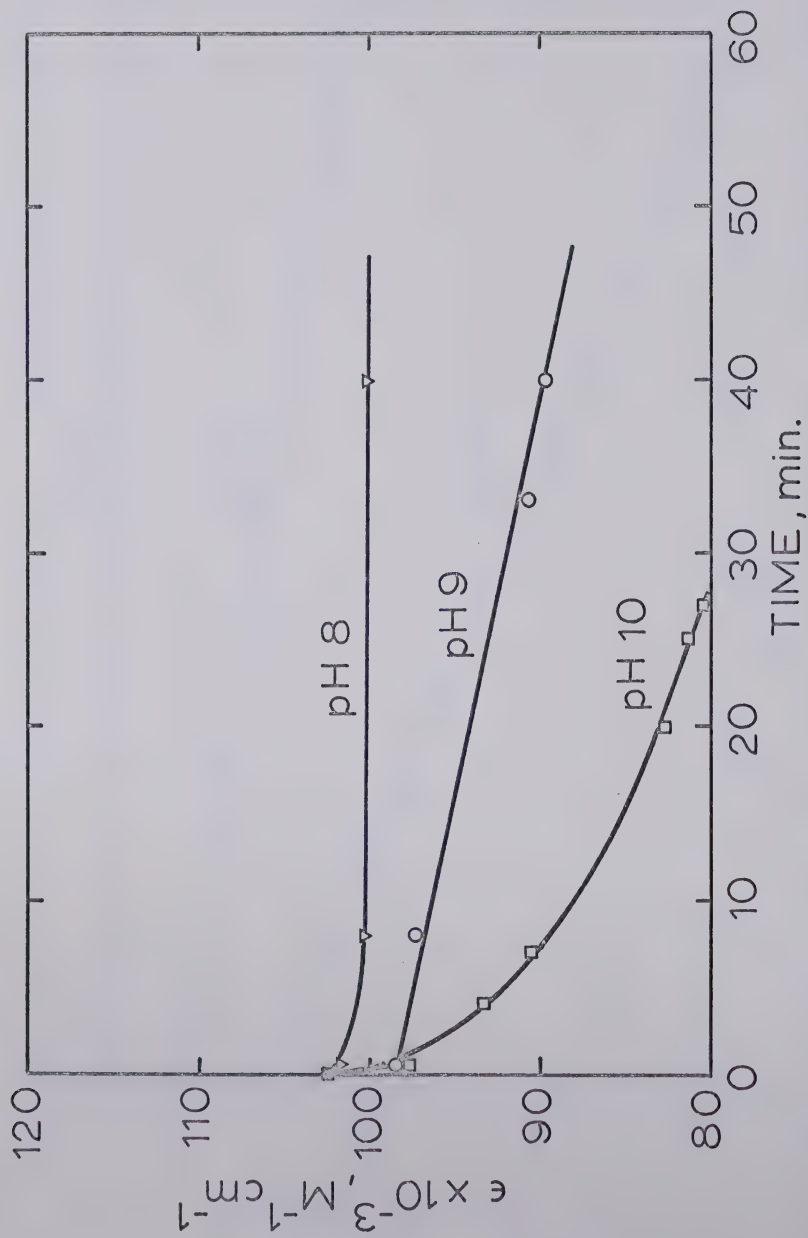


Figure 17. Effect of pH on the absorbance of BrCN-catalase in the Soret region. Apparent ϵ values at 402 m μ . 0.1 M borate buffers, 25°, 4.7 μ M catalase.

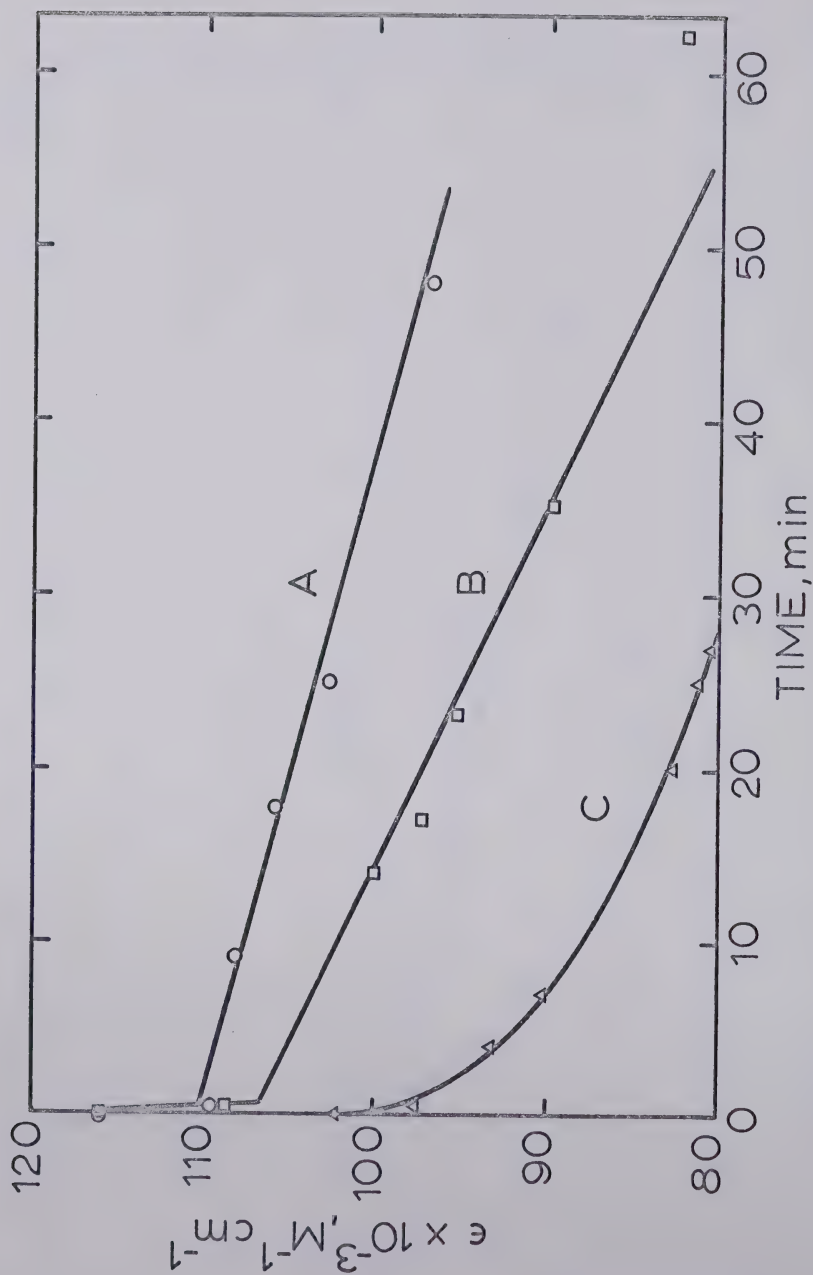


Figure 18. Effect of pH on the absorbance of native, pretreated and BrCN-catalases in the Soret region. Apparent ϵ values at the indicated wavelength. Curve A: native catalase, 405 mμ; Curve B: pretreated catalase, 405 mμ; Curve C: BrCN catalase, 402 mμ. 0.1 M borate buffer, pH 10.0, 25°, 4.7 to 5.5 μ M catalase.

TABLE XVI

Reaction of Pretreated Catalase with BrCN at pH 7 to 10

pH	$\Delta\epsilon, \text{M}^{-1} \text{cm}^{-1}$	$V_{\text{initial}}, \text{M sec}^{-1}\text{^a}$	$k, \text{M}^{-1} \text{sec}^{-1}$
7.0	-	-	142 ^b
8.6	$\sim 23 \times 10^3$	1.0×10^7	145
9.2	$\sim 16 \times 10^3$	1.1×10^7	160
10.0	$\sim 13 \times 10^3$	1.1×10^7	160

0.1 M phosphate at pH 7, 0.1 M borate buffers at other pH values,
25°, 6.2 μM catalase, 110 μM BrCN.

^a Measured 5 - 10 seconds after BrCN addition.

^b Calculated by using graphical first order plot.

were soon dispelled. The limiting factor was the instability of both pretreated and inhibited enzymes (Figs. 16 and 17) which, though not so marked with the native enzyme, became evident at pH 10 (Fig. 18).

Under these circumstances, it would be futile to try to evaluate kinetics of inactivation from spectrophotometric measurements using integrated forms of either first or second order equations. The desired solution could have been achieved using fast reactions techniques, but since this necessitated greater utilization of enzyme, it seemed preferable to explore the reaction by the 'initial velocity' method. The results so obtained are based on the assumption that after 1.5 min. of mixing enzyme and buffer the rate of 'non-specific' absorbancy changes is smaller than the rate of inactivation. Therefore, if the inhibitory reaction is begun after a short (~90 sec.) preincubation of catalase, it seemed that a reasonable estimate of k could be obtained from:

$$v_{\text{initial}} = \frac{\Delta (\text{conc. of inhibited enzyme})}{\Delta (\text{time})} = \frac{\Delta A}{\Delta \epsilon \Delta t}$$

$$\text{where } \Delta \epsilon_{410} = \left(\epsilon_{\text{pretreated}}^{\text{extrapolated}} - \epsilon_{\text{inhibited}}^{\text{extrapolated}} \right)$$

In view of these difficulties, the values of k is not better than about $\pm 25\%$. The results obtained at the alkaline pH values (see Table XVI), indicate that the rate of inactivation is independent of pH up to pH 10. This fact was unexpected, but at least three interpretations are possible:

- a) reaction takes place at a non-ionizing residue
- b) the ionization state of the reactive group does not change in the investigated pH range, and

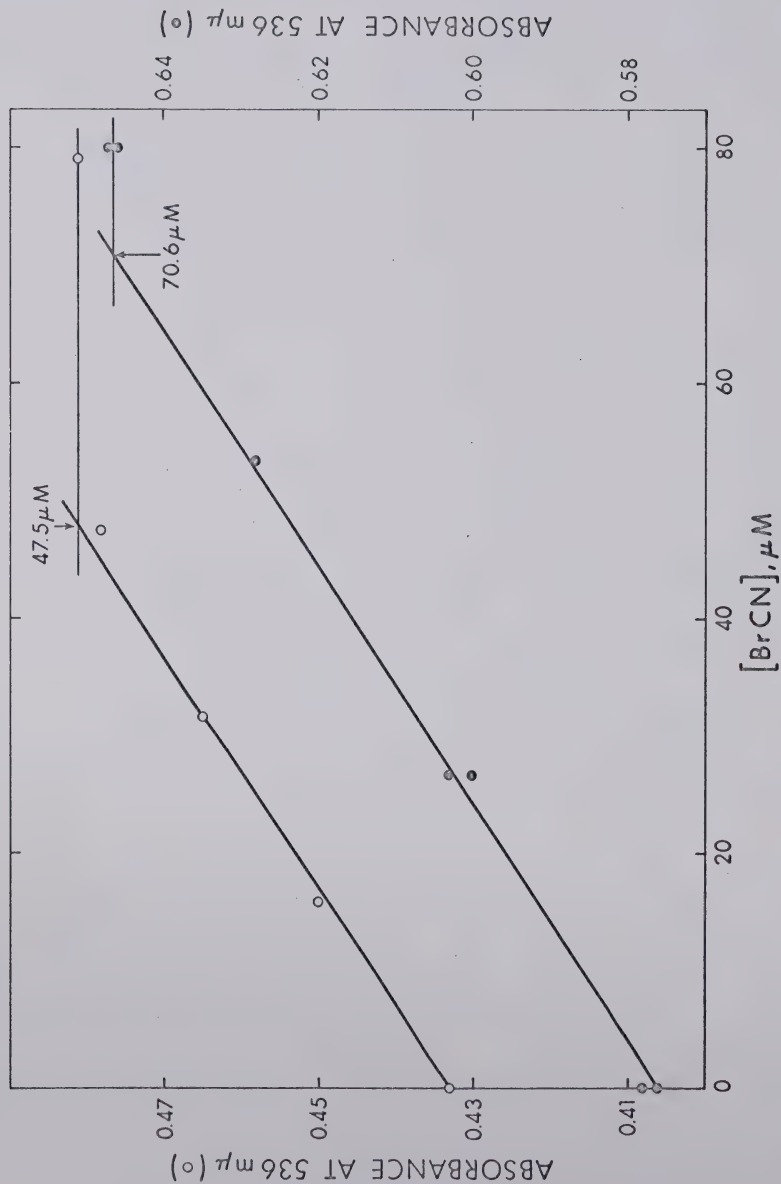


Figure 19. Spectrophotometric titration of pretreated catalase with BrCN. 0.1 M phosphate, pH 7.0, 25°. 44 μM (o), and 59 μM (•) catalase.

TABLE XVII

Titration of Pretreated Catalase with BrCN

[Cat·Fe], μM	Endpoint ^a	$\frac{[\text{BrCN}]}{[\text{Cat}\cdot\text{Fe}]}$
	[BrCN], μM	
58.5	70.6	1.20
43.9	47.5	1.08

0.1 M phosphate, pH 7.0, 25°.

^a From Fig. 17.

TABLE XVIII

Preparation of Br¹⁴ CN-Catalase

Sample No.	Inhibition		Dialysed solution ^d			
	Formed ^a		[Cat·Fe]		[Br ¹⁴ CN-cat]	
	μM	[BrCN-cat], μM	μM	[Br ¹⁴ CN]	μM	[¹⁴ C incorp.] [¹⁴ Br CN-cat.]
1 ^b	76.4	136	-	-	78.4	74.5
2 ^b	76.4	183	-	-	71.6	67.2
3 ^c	87.4	87.3	84.0	1.04	88.0	79.3
4 ^c	85.5	94.0	80.0	1.17	88.6	75.6
5 ^c	67.2	66.0	-	-	68.5	63.5

0.01 to 0.05 M phosphate, pH 7.0 to 7.2, 25°.

^a Estimated spectrophotometrically.

^b PCMB-treated catalase.

^c Pretreated catalase.

^d After the inhibition was complete, the Br¹⁴ CN-catalase solutions were dialyzed against six liter portions of 10 mM phosphate, pH 7, over a period of 42 to 50 hours.

- c) proton transfer attending the modification does not occur in the rate limiting step.

The elimination of any of these possible explanations has to wait until further experimental evidence is given.

Stoichiometry of the catalase-BrCN reaction

The availability of an enzyme devoid of highly reactive sulfhydryl groups permitted now the establishment of the stoichiometry of the inhibition. Two methods were used toward this end; one was based on the spectroscopic differences between an active, pretreated enzyme and its inhibited derivative; the other utilized ^{14}C labelled cyanogen bromide.

In the spectrophotometric procedure, small aliquots of cyanogen bromide were added to a solution of pretreated catalase (44 or 59 μM) and, following completion of the reaction, the spectra were recorded between 450 and 700 $\text{m}\mu$. In this manner further evidence was provided that side reactions do not occur since no deviations were noted at the isosbestic wavelength of 562 $\text{m}\mu$.

These experiments, further detailed in Tables XVII and XVIII and in Fig. 19, lead to the conclusion that irrespective of the initial ratio of BrCN to catalase-hematin only one moiety is modified in the course of reaction. The data left unanswered, however, general questions as to the locus of the reaction (protein vs. hemin); nor was it clear whether bromine radical is retained in the derivative - in addition to CN group. The research was therefore oriented with these questions in mind.

TABLE XIX

Heme Removal from Br¹⁴CN-Catalase

Sample No.	Br ¹⁴ CN-Catalase		After Separation			
	Amount of Cat·Fe, mole	Amount of ¹⁴ C, dpm	In aqueous phase		In organic phase	
			dpm	%	dpm	%
2	13.6×10^{-8}	6.26×10^5	6.04×10^5	97	0.05×10^5	1
4	2.21×10^{-8}	8.40×10^4	7.85×10^4	93	0.39×10^4	5

Sample No.'s correspond to those in Table XVII.

For procedure see text.

Removal of hemin from BrCN-modified catalase

The splitting of a hemoprotein into its components - apoprotein and coenzyme - can be rapidly achieved providing that the prosthetic group is not linked covalently to a protein residue. Such is the case with catalase. Several procedural variants are available but all exploit an increased solubility of metalloporphyrin in organic solvents when pH of the aqueous phase is lowered to 2.5 ± 1.0 . Typically, one can use either acid-acetone method due to Theorell (137) or 2-butanone procedure introduced by Teale (138); the latter was adopted in this work. Experimentally, it involved acidification of catalase - containing one equivalent of ^{14}C per hemin - to pH 2, followed by extraction of ferriprotoporphyrin with equal volume of 2-butanone. Upon separation of organic (deep brown) and aqueous (colourless) phases (2 - 3 min.), aliquots were withdrawn from each layer and radioactivity estimated as documented previously (see Ch. II). The data, in Table XIX shows unequivocally that almost the entire radioactivity associated, originally, with the inhibited catalase remains in the aqueous layer containing apoprotein. This demonstration would have constituted a convincing proof of protein involvement in BrCN reaction but for the reservations:

- a) that the derivative undergoes rapid hydrolysis to give products almost entirely insoluble in the organic phase; or
- b) that radioactive moiety had been transferred from hemin to apoprotein in the course of enzyme splitting.

Neither postulate is likely. To substantiate this statement, consider these additional facts:

1. The radioactive compound remaining in aqueous solution is not stable at pH 2. It decomposes slowly*, to give $^{14}\text{CO}_2$ in 83% yield relative to the original radioactivity (see page 69). Therefore, ^{14}C -containing moiety could not have been transferred to a methionine or some masked sulfhydryl remaining in the pretreated enzyme, since methionine should rearrange to give methyl thiocyanate (135) and thiocyanates are not expected to hydrolyse under mild acid conditions. Other potential nucleophiles are unreactive at low pH and preformed compounds such as $-\text{NH}-\overset{\overset{^{14}\text{C}}{\parallel}}{\text{O}}-\text{NH}_2$ should not decompose at pH ~ 2 .
2. Carbon dioxide would be expected if cyanate were released into the medium but, since radioactive compound cannot be expelled from the solution immediately following acidification, therefore cyanic acid - and hence CO_2 - are not appreciably formed with the span of 2 - 5 min. necessary for fission of the holoenzyme.

* The pH of a sample of Br^{14}CN -catalase, prepared by using stoichiometric amounts of Br^{14}CN , was lowered to 1.8 by adding concentrated HCl. This acidified solution was placed into an open container and was stirred continuously at 25° . Samples were removed at suitable time intervals (0.5 to 3 hours) to estimate the radioactivity remaining in the solution. The half time of the disappearance of ^{14}C from the solution was found to be 80 ± 20 minutes in three experiments.

TABLE XX

Catalase Preparations used for Neutron Activation Analysis

Preparation	Total [Cat·Fe], μM	BrCN-Catalase		Activity	
		μM	%	$k \times 10^{-5}, \text{M}^{-1} \text{sec.}^{-1}$	%
A	90.2	0	0	116	100
B	92.1	1.8	2.0	88.2	76.2
C	93.6	47.0	50.2	55.6	48.0
D	72.4	70.2	97.0	4.1	3.5
E	65.3	~64.6	~99	1.1	1.0

All preparations were extensively dialysed against 50 mM ammonium citrate, pH 6.6.

TABLE XXI

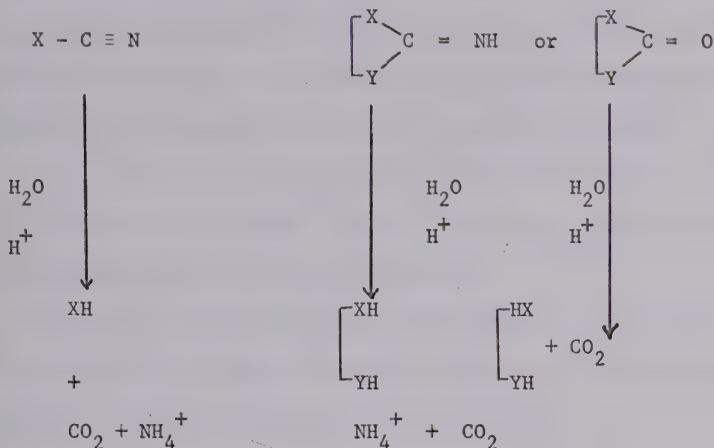
Neutron Activation Analysis for Bromine in Catalase Preparations

Sample No.	Catalase Preparation ^a	Final [Cat·Fe], μM	Final [BrCN-Cat·Fe], μM	Added [Br ⁻], μM	Found [Br ⁻], μM	Error \pm %
1	B	77.6	1.6	0	2.9	14
2	"	"	"	8.4	8.9	4.4
3	"	"	"	16.8	15.4	3.3
4	"	"	"	42	48.8	1.8
5	"	"	"	84	88.4	1.4
6	C	78.1	39.2	0	<1.2	-
7	"	"	"	8.4	9.9	4.1
8	"	"	"	42	45.8	1.9
9	"	"	"	84	84.5	1.5
10	D	60.2	58.4	0	<1.7	-
11	"	"	"	8.4	9.7	4.4
12	"	"	"	42	43.6	2.0
13	"	"	"	84	89.8	1.5
14	E	54.4	53.8	0	<1.7	-
15	"	32.6	32.2	0	1.6	18
16	Buffer only	-	-	0	<1.0	-
17	- " -	-	-	8.4	8.4	3.6
18	- " -	-	-	42	42.0	1.8

All samples were prepared by using 50 mM ammonium citrate, pH 6.6, as diluent.

^a Data about these preparations are given in Table XIX.

On this basis, the possibility of formation of ^{14}C compound other than in combination with protein is discounted, and the minimal structure therefore must be



where X and Y are the functional groups of protein backbone.

It becomes of interest now to enquire whether Br is also a constituent of such a derivative.

Bromine content of BrCN-modified catalase

Although assay of bromide does not present undue difficulties above 10^{-4} level, the conversion of potential organic halogen compounds into a form suitable for standard analytical techniques seemed a complex undertaking. Moreover, since only limited amounts of enzyme were available, it was essential to resort to a technique which would not depend on the state of 'bromide' compound but would give reliable results, even at 0.1 ppm ($\sim 10^{-6}$ M Br). Such a method is neutron activation analysis. The assays, performed by Gulf General Atomic Inc. (San Diego), were carried out on eighteen samples and included the necessary controls (Tables XX and XXI).

To enhance the sensitivity of the analysis, all preparations were extensively dialyzed against 50 mM ammonium citrate to remove alkali metal halides and phosphates, since these compounds could interfere with analysis of bromine at ppm level. The reliability of the method is clearly substantiated by the results obtained with samples to which ammonium bromide was intentionally added.

It is seldom that one can make an unqualified statement; this is one of those rare occasions. In short, Br is not bound covalently in the pretreated or inhibited catalases.

Barring then the possibility of salt formation, it seemed very likely that proton release would also occur in the course of enzyme inhibition. This inference was shortly confirmed.

Stoichiometry of proton release

Ideally, the experiments should have been conducted over a wide range of hydronium ion concentration, using pH-stat. Due to various technical difficulties, the project had to be deferred; instead, the pH changes attending the reaction of pretreated catalase with cyanogen bromide were monitored using a Radiometer PHM 25 connected to an Esterline Angus recorder. Since both instruments had exceptional electronic stability, it was possible to follow even small pH changes, providing the temperature of the reaction vessel varied by less than 0.1° and precautions were exercised to prevent diffusion of gases (CO_2 , NH_3). The experiments were carried out with salt free solutions of pretreated catalase whose buffering capacity between pH 5.8 and 6.8, was calibrated with standardized

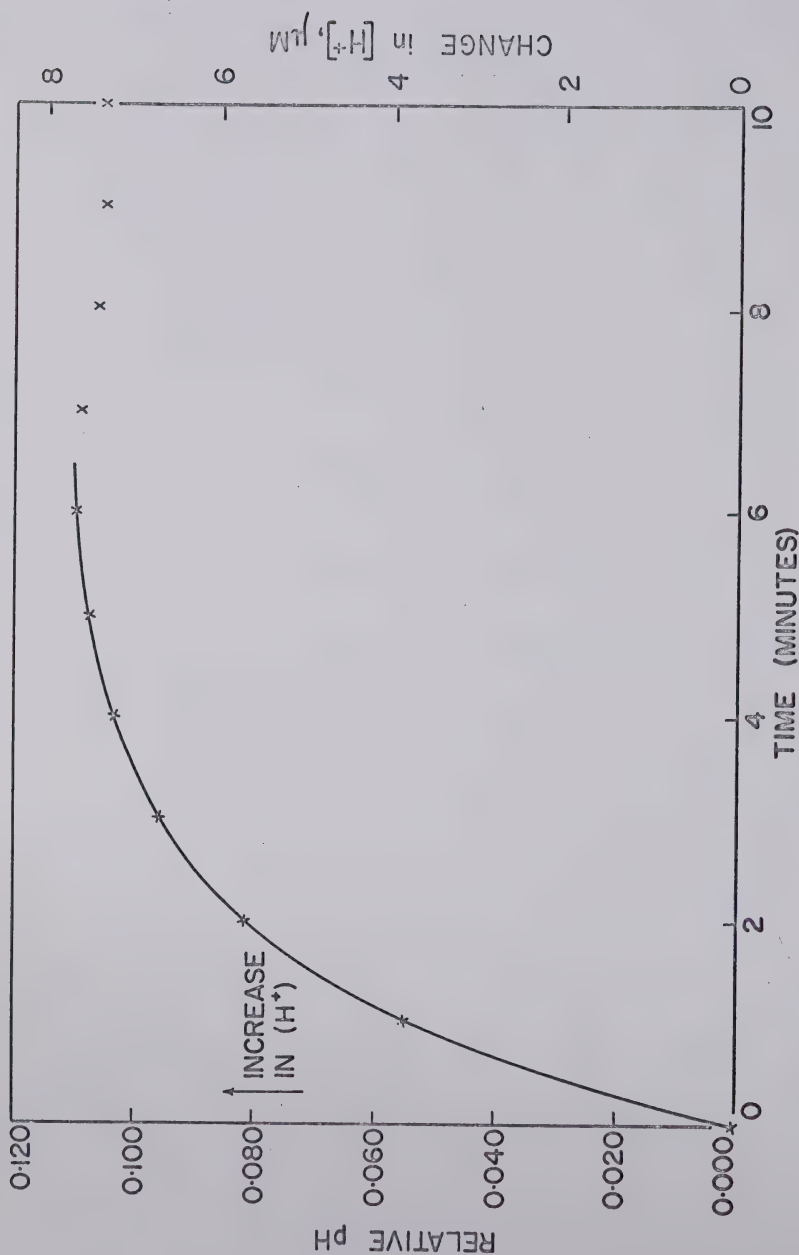
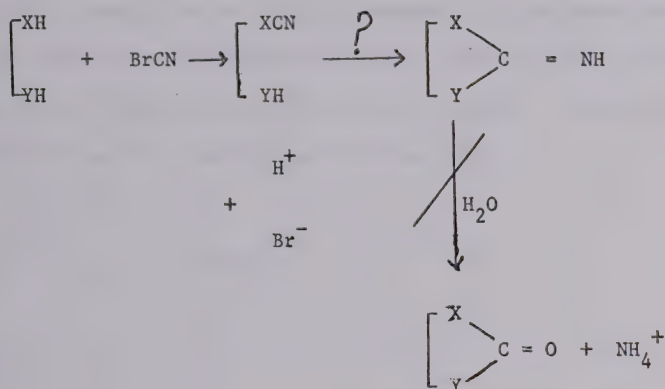
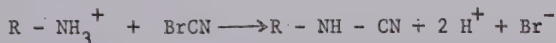


Figure 20. Proton liberation during the inhibition of pretreated catalase by BrCN. Glass distilled water, $25.0 \pm 0.1^\circ$; $9.8 \mu M$ catalase, $74 \mu M$ BrCN. Measurements were made in the pH range of 5.9 - 6.2.

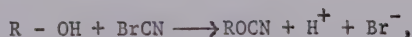
HCl and NaOH. Cyanogen bromide was then added via small port in the sleeve of the reaction vessel and the resulting decrease of pH was recorded. The result of such an experiment, given in Fig. 20, shows that approximately 0.8 mole of H^+ is released per mole of modified catalase-hematin, and suggests that ammonia is not formed in the inhibitory reaction:



Nor is it likely that a lysine, proline or α -amino residue enters into the reaction since greater proton liberation would be expected in such a case.



The remaining possibilities include formation of cyanate or thiocyanate esters:



N-cyanation of imidazole ring of histidine:



or cyanation of guanidine residue of arginine.

The instability of 'CN-catalase' following its denaturation at pH 2 is not readily interpreted if cyanate, or thiocyanate esters are the derivatives. Rather arbitrarily, therefore, the choice would appear to lie between histidine, or arginine (and forsaken - tryptophane) as the possible locus of reaction.

CHAPTER IV

Physical and chemical properties of native, pretreated and BrCN-modified catalases

In the first part of this Chapter a comparative study will be made on the three types of catalase preparations with respect to certain physical and chemical properties; in the second portion of the Chapter additional properties, unique only to the inhibited enzyme, of the BrCN-modified enzyme will be described.

It has to be mentioned at this point that all preparations used in the studies described in this Chapter were periodically checked (enzymatic activity, absorption spectra) to ensure that no changes occur in their properties during storage. At any rate, BrCN-modified catalase was found to be stable for several weeks when stored at 4°.

The preparation of pretreated catalase is given in Chapter III. BrCN-modified catalase (BrCN-catalase) was generally prepared by adding about 10 to 30% excess (with respect to hematin-Fe) BrCN to pretreated catalase in 10 mM phosphate buffer, pH 7, at 25°. The reaction was monitored spectrophotometrically until no further change was observed and then the solution was dialyzed against the same phosphate buffer. In the case of some preparations, large excess or less than stoichiometric amounts of BrCN were used for the inhibition under the above mentioned conditions, and was followed by dialysis. In these cases the [BrCN]/[catalase] ratio used in the modification procedure is specifically stated so its relevance to the results is not overlooked.

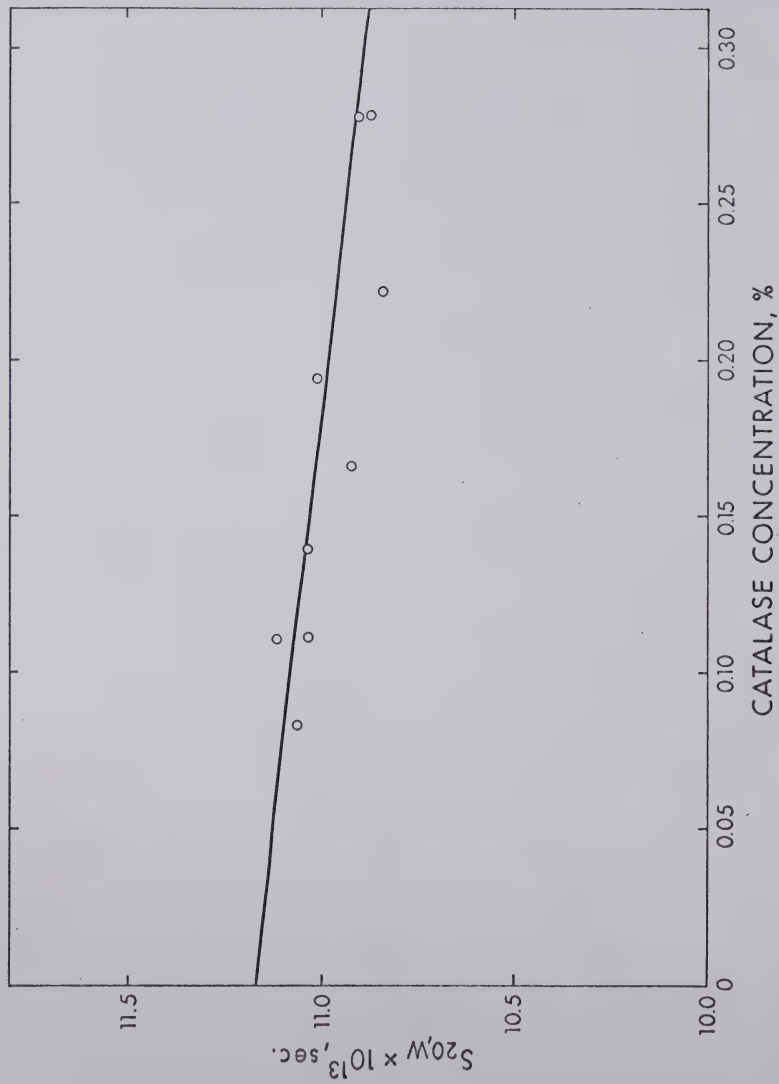


Figure 21. The concentration dependence of the sedimentation constant of native catalase. 0.105 M phosphate, pH 7.0, $20.0 \pm 0.1^\circ$, 60,000 r.p.m.

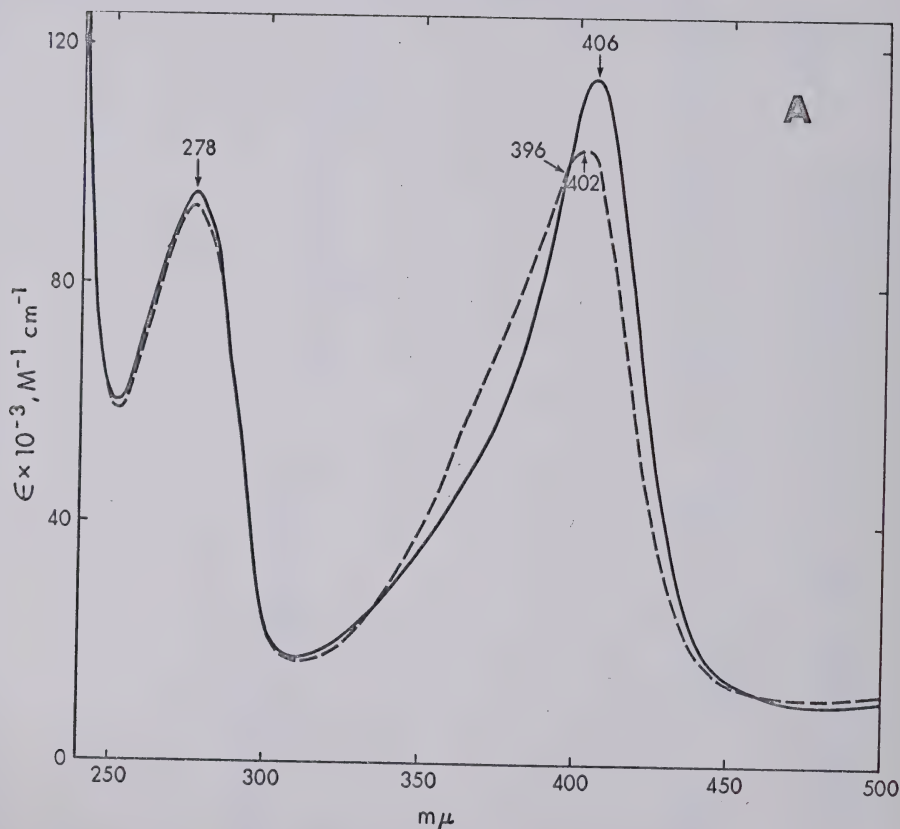
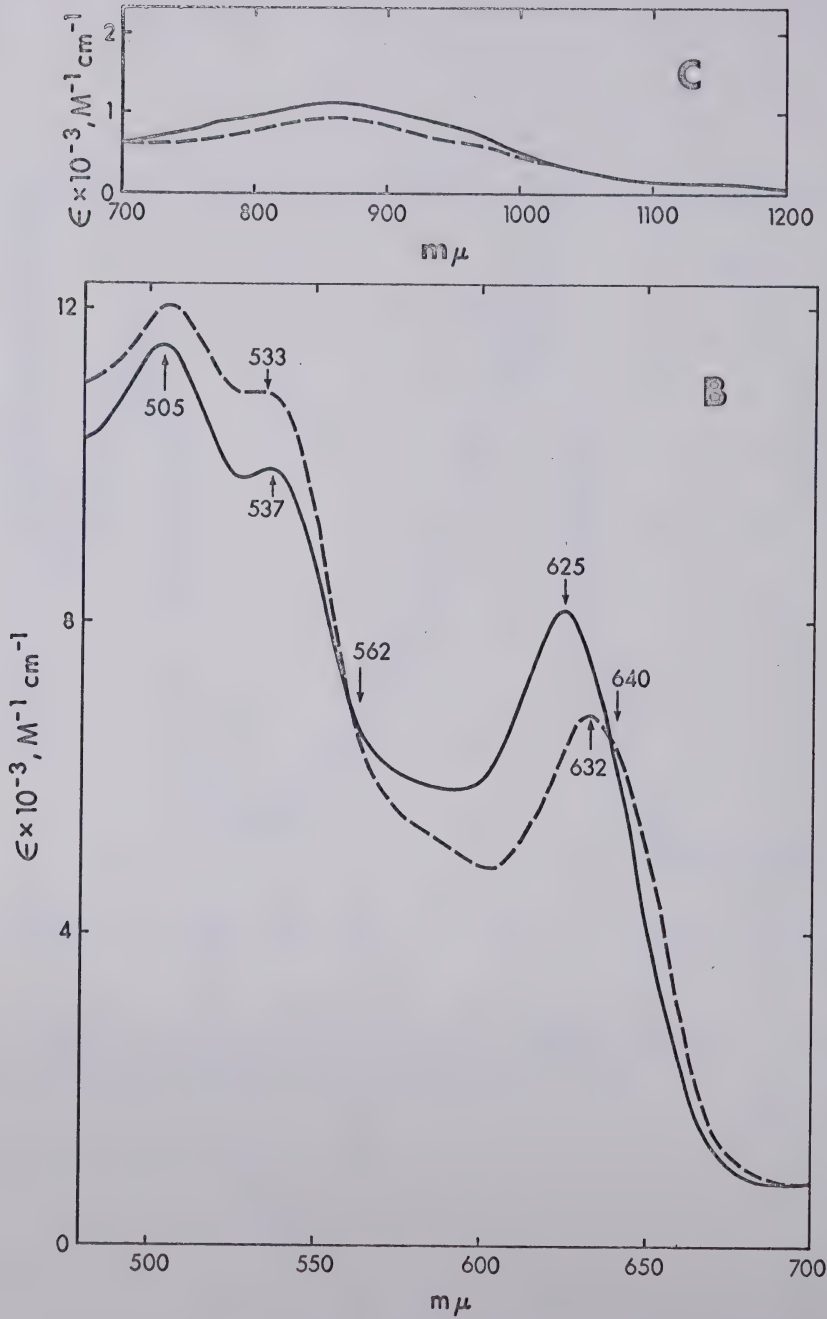


Figure 22. Absorption spectra of native catalase (—) and its BrCN derivative (---) in the UV (A), visible (B) and near IR (C) regions. 0.1 M phosphate, pH 7.0, 25°.



"Figure 22"

TABLE XXII

Sedimentation Constants of Native, Pretreated, and BrCN-Catalases

Sample No.	$S_{20,w} \times 10^{13}$, sec.						
	1	2	3	4	5	6	7
Native Catalase	11.06	11.12	11.12	11.16	11.16	-	-
Pretreated Catalase	11.12	11.09	11.12	11.06	11.21	11.06	11.09
BrCN-Catalase	11.06	11.16	11.06	11.09	11.12	-	-
							Average
							11.12 \pm 0.06
							11.11 \pm 0.10
							11.11 \pm 0.05

0.105 M phosphate buffer, pH 7, 20.0°.

Native catalase: 35.3 μ M, or 0.220 %.Pretreated catalase: 35.4 μ M, or 0.221 %.BrCN-catalase: 33.9 μ M, or 0.212 %.

Hydrodynamic properties and optical absorption spectra of native, pretreated and BrCN-modified catalases

In order to get reliable results for the different catalase preparations, the sedimentation constant of native catalase was determined at a number of different concentrations. The results are plotted on Fig. 21; the line was fitted to the experimental points by the least square method with the aid of a computer program. The extrapolation of the line to zero concentration gives a value of 11.2 ± 0.05 S for $s_{20,w}^0$.

Since in the case of native catalase the sedimentation constant did not show a marked concentration dependence, the sedimentation properties of the three catalase preparations were determined at a single protein concentration. The results obtained are presented in Table XXII; they show that gross structural changes do not attend the pretreatment and BrCN-modification of the native enzyme.

Similarly, the absorption spectra of native and BrCN-modified catalases shown only marginal differences in the ultraviolet and although there are some characteristic changes in the Soret, visible, and near IR regions, even these are not suggestive of drastic alteration of the protein structure (Fig. 22). The absorption spectrum of pretreated catalase is not shown in Fig. 22, because it was found to be identical to that of the native enzyme within the experimental error of the spectrophotometric measurements.

Circular dichroism measurements were utilized to detect subtle conformational changes that could attend the pretreatment and the inhibition of the native enzyme. The ultraviolet circular

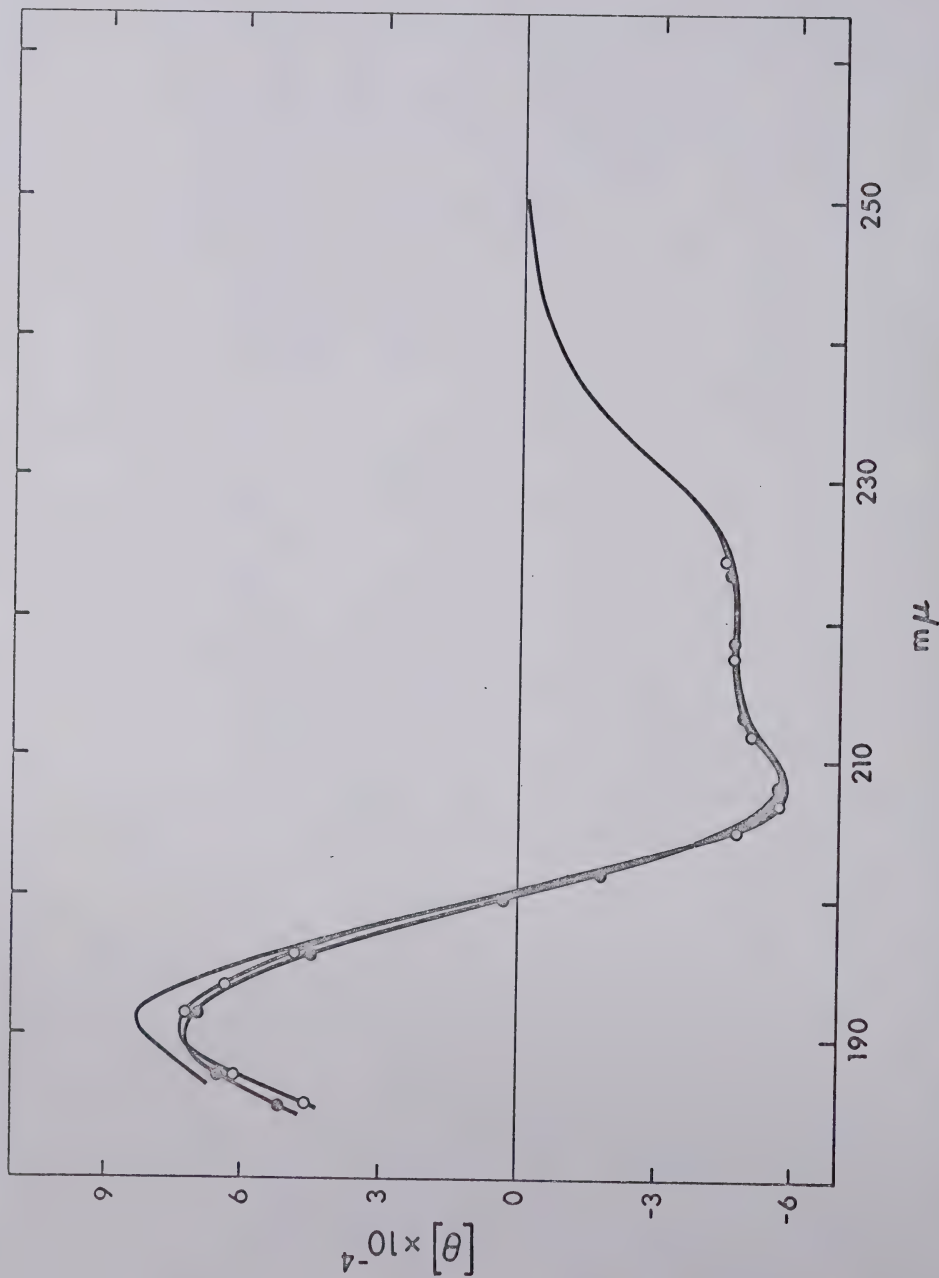


Figure 23. Ultraviolet circular dichroism spectra of native (—), pretreated (—○—) and BrCN catalases (—●—). See text for experimental details. Molar ellipticities $[\theta]$ were calculated by using heme concentrations, and have the dimensions of degrees $\cdot \text{cm}^2$ per decimole.

dichroism spectra of native, pretreated, and BrCN-modified catalases are presented in Fig. 23; the measurements were made in 10 mM phosphate buffer, pH 7, at 25°; the concentration of hematin-Fe was 12.5 μ M in all three solutions.

Since the determination of inherent enzyme helicity was not the principal aim of this work, comments will be restricted to comparative values of $[\theta]^a$, as outlined in Fig. 23. The deviations in $[\theta]$, at 190 m μ , among the three preparations are ascribed to technical shortcomings rather than to differences in peptide interactions within the protein. Significantly, corresponding changes were not observed at 208 m μ , although both bands have origin in $\pi-\pi^*$ transitions of the carbonyl group.

The inhibition cannot arise therefore from an extensive disruption of the tertiary or quaternary protein structure, and it is tempting to speculate that even minor changes are not the contributory cause. The remaining possibilities encompassed steric inhibition and a more interesting case of specific modification of an essential functional group. The distinction between these alternatives is perhaps one of the most difficult tasks and short of recourse to x-ray crystallography any argument must remain of a tentative nature. The finding that various potential ligands can

$^a \text{Molar ellipticity } [\theta] = \frac{\theta_{\text{obs}}}{10 \text{ l c}}$	where θ observed ellipticity l optical cell path (cm) c hemin conc. in moles/ml.
--	--

TABLE XXIII

Amino Acid Composition of Native,
Pretreated and BrCN-modified Catalases^a

Amino acid	Native	Pretreated	BrCN-modified
Ala	165	165	165
Arg	130	130	130
Asp	302	309	307
Cys acid ^b	24	23	23
Glu	213	214	216
Gly	148	143	145
His	75	74	73
Ileu	83	85	84
Leu	147	151	142
Lys	129	126	123
Met	45	45	44
Met-sulfone ^b	44	42	44
Phe	135	137	134
Pro	193	188	178
Ser ^c	106	102	105
Thr ^c	104	104	106
Try	-	-	-
Tyr	80	80	79
Val	159	160	149

^aNumber of amino acid residues per mole of catalase.

^bDetermined according to Moore (56).

Cysteic acid values were corrected for 6% hydrolytic loss.

^cExtrapolated values at zero time.

effect partial reactivation of the cyanated enzyme was therefore particularly important. It suggested that the reactants can still diffuse to the active site, even if they do not necessarily interchange or bind at the sixth coordination position. It becomes thus less likely that inhibition is entirely attributable to steric interference at the distal region of ferriprotoporphyrin.

Amino acid composition of native, pretreated and BrCN-modified catalases

The results of the amino acid analyses are given in Table XXIII, and they indicate that neither pretreatment nor the BrCN-modification causes a significant change in the amino acid composition of the native enzyme.

Comparison of the values obtained for horse blood catalase in this study with those given in Table IV shows some differences in amino acid compositions, keeping in mind, of course, that variations are present between the same enzymes from different species.

Sulfhydryl groups of native, pretreated and BrCN-modified catalases

The sulfhydryl groups of the three catalase preparations were estimated by the procedure described in detail in Chapter II.

The denaturation of the enzyme preparations was achieved by lowering the pH to 1.5 to 1.8; after three hours of incubation at 25°, the pH of the solution was readjusted to 4.6 ± 0.1 .

TABLE XXIV

-SH Group Estimation of Native, Pretreated and BrCN-Catalases

Sample No.	Catalase Preparation	No. of -SH Groups / Catalase Molecule	
		Undenatured	Acid-Treated
1	Native	14.3	17.0
2	-- " --	14.2	17.9
3	Pretreated	6.1	-
4	-- " --	4.4	7.9
5	-- " --	2.3	-
6	BrCN-modified	3.7	-

0.33 M acetate, pH 4.6, 25°.

The number of -SH groups that were titratable by PCMB in the undenatured and acid-treated preparations is given in Table XXIV. The values shown are the averages of at least two determinations.

The number of sulfhydryl groups found in undenatured and acid-treated native catalase (samples 1 and 2 in Table XXIV), and the fact that there are 24 half-cystine residues (from Table XXIII) in the enzyme, do not permit a definite statement about the number of cystine residues in horse blood catalase, since the data could accommodate the presence of only three disulfide bonds. This reservation is particularly meaningful because current proposals suggest that catalase is composed of four identical subunits (for data and references, see Table II). On the other hand, the work of Samejima and Yang (105) and that of Schroeder et al. (57) raised the possibility that the enzyme is devoid of disulfide bonds. This contention is supported by the fact that acid-denatured catalase appears to retain some tertiary structure, as indicated by circular dichroism measurements (125), and -SH groups within this core are probably not available for titration by PCMB. Furthermore, cystine residues could derive from the adventitious oxidation of cysteines during the isolation procedure or storage.

The results of PCMB titrations of pretreated and inhibited catalases posed new problems (Table XXIV). At first, it was surprising that on pretreating catalase with cyanogen bromide any sulfhydryl groups, which could be titrated with PCMB, escaped oxidation by BrCN. Accessibility cannot be a factor, because undenatured protein gives mercaptides with PCMB. Moreover, different

preparations of the pretreated enzyme retained variable susceptibility to PCMB. This can only mean that not all catalase sulfhydryls are oxidized equally; a point which was not appreciated in devising the pretreatment method (page 46).

Even more astounding is the retention of PCMB reacting groups in the inhibited enzyme. Taken at its face value and recalling the excellent, 1:1, stoichiometry of the specific inhibition, the following picture seems to be emerging. Catalase contains, at least, 17-18 sulfhydryl groups (data with acid treated native catalase); of these, fourteen can enter into mercaptide formation with PCMB, but only eight are rapidly oxidized by cyanogen bromide. The remaining six residues are not inert to oxidation but the rate of reaction must be substantially lower. Therefore, short of specific activation, it is unlikely that these sulfhydryls participate directly in the catalytic process. To do so their redox turnover must be consonant with the rates of formation and decomposition of compound I, or some $5 \times 10^6 \text{ M sec}^{-1}$.

Since preincubation was carried out for at least three hours, using $\sim 10^{-4} \text{ M CatFe}$ and $\sim 2 \times 10^{-3} \text{ M BrCN}$, therefore the velocity constant for oxidation of "slow" SH groups must be smaller than $10^{-2} \text{ M}^{-1} \text{ sec}^{-1}$, i.e. some 8-9 powers of ten smaller than the constant for compound I formation.

Of course, like any other intuitive interpretation, the above analysis cannot be more than suggestive. A stronger case could have been made if greater elimination of sulfhydryls were achieved.

TABLE XXV

Catalatic Activity of Pretreated Catalase

Sample No.	No. of Determinations	Final [Catalase] $\times 10^9$, M	$k_1^1 \times 10^{-7}$ $M^{-1} \text{ sec.}^{-1}$
1	3	2.20	1.06
2	6	1.95	1.00
3	21	1.91 to 3.20	0.98
4	7	1.93 to 3.22	0.96
5	6	2.20	1.01
6	3	2.88	0.91
7	3	2.80	0.90
8 ^a	6	1.78	1.08

5 mM phosphate buffer, pH 7, 25°.

~8 mM H_2O_2 .

^a PCMB-treated catalase (see text).

In two respects, at least, the presence of some reduced thiol groups is not essential. Thus, both the tetrametric structure and the helicity of the enzyme remain unaltered either on pretreatment or inhibition of catalase.

Catalatic activities of native, pretreated, and BrCN-modified catalases

The catalatic activities were determined under the standard conditions given in Chapter II. In Table XXV the catalatic activities of pretreated and PCMB-treated* enzyme preparations are given. Comparison of these values with the activity of native catalase (see Table VII) indicates that neither PCMB-treatment nor the pretreatment influences the catalatic activity of the enzyme, and this can be taken as evidence that the fast reacting -SH groups (up to 14 per catalase molecule, see Table XXIV), which can be titrated by PCMB, do not play any role in the catalytic function of the enzyme. No such statement can be made about the 3 to 4 sulfhydryl groups, which become titratable by PCMB upon acid denaturation of the enzyme, but the results to be presented on the regeneration of BrCN-modified catalase (see page 67) are incompatible with a modified -SH group in the inhibited enzyme and thus it is very unlikely that these buried sulfhydryl groups are involved in the enzymatic function of catalase (see also page 15).

* 86 μ M catalase was treated with 760 μ M PCMB in 0.1 M acetate, pH 4.5, for 12 hours at room temperature. Excess PCMB was not dialyzed off before activity measurement.

TABLE XXVI

Catalatic Activity of BrCN-Catalase

Sample No.	No. of Determinations	$\frac{[\text{BrCN}]^a}{[\text{Catalase}]}$	Final [Catalase] $\times 10^8$, M	$k_1^1 \times 10^{-5}$ $\text{M}^{-1} \text{sec.}^{-1}$
1	6	3	56.4	0.6
2	32	1.2	7.0 to 13.0	3.4
3	12	1	4.31	6.7
4	3	1	3.80	8.1
5	3	1	14.7	3.4
6	6	1.5	4.0 to 35.6	0.9
7	6	1.3	7.39	2.6
8	3	1.1	6.26	3.6

5 mM phosphate buffer, pH 7, 25°.

~8 mM H_2O_2 .

^a Condition during modification procedure.

TABLE XXVII

Catalatic Activity of Various BrCN-Catalase Preparations

Sample No.	[BrCN] ^a [Catalase]	% BrCN-Cat. ^b Formed	Final [Catalase] x 10 ⁸ , M	$k_1 \times 10^{-5}$ M ⁻¹ sec ⁻¹	% Activity
1 ^c	-	~0	0.28	90.0	100
2	0.3	28	0.34	64.1	71
3	0.6	52	0.56	41.2	46
4	0.8	77	0.70	22.4	25
5	1.0	89	1.38	9.6	11
6	1.0	93	2.69	6.1	7
7	~2	over 99	53.6	0.5	0.6

5 mM phosphate, pH 7, 25°.

~8 mM H₂O₂.

^a Condition during modification procedure.

^b Estimated spectrophotometrically.

^c Pretreated catalase sample.

TABLE XXVIII

Effect of Repeated BrCN-Treatment on the
Catalatic Activity of Pretreated Catalase

Sample	$\frac{[\text{BrCN}]^a}{[\text{Catalase}]}$	No. of Determinations	Final [Catalase], M	$k_1 \times 10^{-5},$ $\text{M}^{-1} \text{sec.}^{-1}$
Pretreated Catalase	-	6	2.32×10^{-9}	92.0
First BrCN-treatment	1.5	9	7.75×10^{-8}	1.6
Second -- " --	~ 12	3	1.00×10^{-6}	0.11
Third -- " --	~ 12	3	1.00×10^{-6}	0.10

5 mM phosphate, pH 7, 25°.

~ 8 mM H_2O_2 .

^a Conditions during BrCN-treatment.

The catalatic activities of a number of BrCN-modified catalase preparations are given in Table XXVI. The results indicate that at least 90% inhibition occurs when the enzyme is treated with stoichiometric amounts of BrCN. Therefore, not more than 10% of the added BrCN is used up by non-specific side reactions with protein functional groups. When slight excess of BrCN was used (10 to 50%) inhibition up to 99% was achieved.

Table XXVII shows the catalatic activities of a number of BrCN-catalase preparations, which were obtained by using less than stoichiometric amounts of BrCN for the modification. The results show good correlations among the amount of BrCN added, the amount of BrCN-catalase formed, and the activity of the modified enzyme. These findings provide definite evidence for the fact that the spectrophotometrically observed changes upon BrCN-modification are directly related to the loss of enzymatic activity.

Examining the data in Table XXVIII it is clear that, even on repeated exposure to BrCN, the catalatic activity decreases some thousand-fold only. Granted that complete elimination of hydrogen peroxide decomposition would be virtually impossible - since hemo-proteins, hemin and transition metals in general have some catalatic action - the magnitude of the residual activity ($k = 10^4 \text{ M}^{-1} \text{ sec}^{-1}$) was too great to be lightly discussed. Three possibilities were therefore considered:

- a) that enzyme solutions contained some entrained heavy metals

TABLE XXIX

Effect of EDTA on the Catalatic Activity
of Native and BrCN-Catalases

Sample	No. of Determinations	Final [Catalase], M	[EDTA], mM	k_1^1 , $M^{-1} \text{ sec.}^{-1}$
Native	4	1.90×10^{-9}	-	0.89×10^7
Catalase	3	-- " --	0.98	0.88×10^7
BrCN -	2	5.64×10^{-7}	-	7.0×10^4
Catalase	2	-- " --	0.99	6.8×10^4

5 mM phosphate, pH 7, 25°; ~8 mM H_2O_2 .

TABLE XXX

Effect of HCN on the Catalatic Activity
of Native and BrCN-Catalases

Sample	No. of Determinations	Final [Cat.], M	[HCN] ^b , μM	k_1^1 , M ⁻¹ sec. ⁻¹	k_1^1 (no HCN) k_1^1 (with HCN)	
					[HCN] [Catalase]	
Native	2	1.90×10^{-9}	-	1.00×10^7	-	
Catalase	3	-- " --	99	7.50×10^{5a}	5.21×10^4	13.3
BrCN -	4	5.66×10^{-7}	-	5.00×10^5	-	12.1
Catalase	2	-- " --	99	4.13×10^{3a}	3.83×10^{4b}	

5 mM phosphate, pH 7, 25°; ~8 mM H₂O₂.

^a

Calculated on the basis of total catalase present.

^b "Catalase" is taken to be unmodified catalase that could give the observed activity, that is

$[\text{catalase}_{\text{Total}}] \times 0.005$.

- b) that the BrCN-modified derivative retains partial intrinsic activity, and
- c) that the residual capacity for H_2O_2 decomposition is due to an incomplete modification.

The first of these was eliminated by carrying out the assays in the presence of EDTA (Table XXIX). Distinction between the remaining propositions proved far more difficult. Thus, following 99.9% inhibition, the rate of further inactivation becomes so small that to achieve any substantial change, the concentration of the enzyme, or cyanogen bromide, or both, must be increased proportionally. When this is done, the activity drops to 0.01% (relative to the native enzyme) (49), but the result is questionable in view of possible secondary modification.

A simpler way to resolve the problem is inherent in the apparent loss of HCN binding capacity by the cyanated enzyme. Suppose now that the reactivity of native and modified catalases towards H_2O_2 be determined in the presence of equal amounts of cyanide. By choosing the concentrations of both enzymes so that the amount of active species is nearly the same, it was expected that the ratio of the observed rate constants k_1' (no HCN)/ k_1' (with HCN) would remain invariant if cyanated catalase is completely devoid of activity. Data in Table XXX support this hypothesis, suggesting that the residual activity is principally due to some uninhibited enzyme.

TABLE XXXI

The Catalytic Activity of Native, Pretreated and BrCN-modified
Catalases in the Francoeur-Denstedt Reaction

Experiment No.	Catalase Preparation	Final [Catalase], μM	Rate ^a , ΔA_{420} in 10 min.	Corrected rate ^b , ΔA_{420} in 10 min.
1	-	-	0.028	-
2	Native	0.88	0.111	0.083
3	- " -	1.76	0.143	0.115
4	Pretreated	0.59	0.026	-
5	- " -	1.18	0.023	-
6	BrCN-modified	0.55	0.027	-
7	- " -	1.11	0.026	-

25 mM Tris buffer, pH 8.5, 25°.

^aObserved initial rate; formation of ferrocyanide was indicated by the decrease in absorbance at 420 m μ .

^bObserved minus the rate in the absence of catalase (Exp. 1).

Francoeur-Denstedt reaction

The activity of native, pretreated, and BrCN-modified catalases was examined in the Francoeur-Denstedt system. The rate of ferricyanide reduction was followed at 420 m μ in the presence of 18 mM ribose-5-phosphate and 0.8 mM ferricyanide. In Table XXXI the amount of catalase used in each experiment is given. All solutions were purged thoroughly with N₂, and the reaction was carried out in cuvettes stoppered by serum caps. The reaction was initiated by the injection of catalase through the serum caps.

From the results given in Table XXXI it is obvious that not only BrCN-modified catalase but also the pretreated enzyme failed to act as catalyst in the Francoeur-Denstedt system. These findings reinforce the explanation given earlier (see page 18), that in this system the prosthetic group, and its immediate environment, of catalase are not involved in the catalytic process, because the pretreated enzyme, which retains almost full catalatic activity (see page 63), is unable to enhance the rate of ribose-5-phosphate oxidation. It is more likely that the pretreatment of catalase results in the modification of a protein functional group which actually catalyzes the oxidation of the sugar phosphate in the presence of ferricyanide. Since the pretreatment of catalase results in the oxidation of a number of sulphydryl groups, one or more of these could be involved in the catalysis exhibited by native catalase in the Francoeur-Denstedt reaction.

Figure 24. Regeneration of BrCN-catalase. The effect of formate, acetate and fluoride on the catalatic activity of the inhibited enzyme. 39 μ M catalase, pH 4.5, 25°; 0.3 M formate (\bullet), 0.2 M formate (Δ), 0.1 M formate (\circ) 0.095 M fluoride and 5 mM acetate (\square), 0.2 M acetate (∇), and 0.1 M phosphate and 5 mM acetate (\blacksquare). Activities were determined as described previously after appropriate dilution of the above solutions.

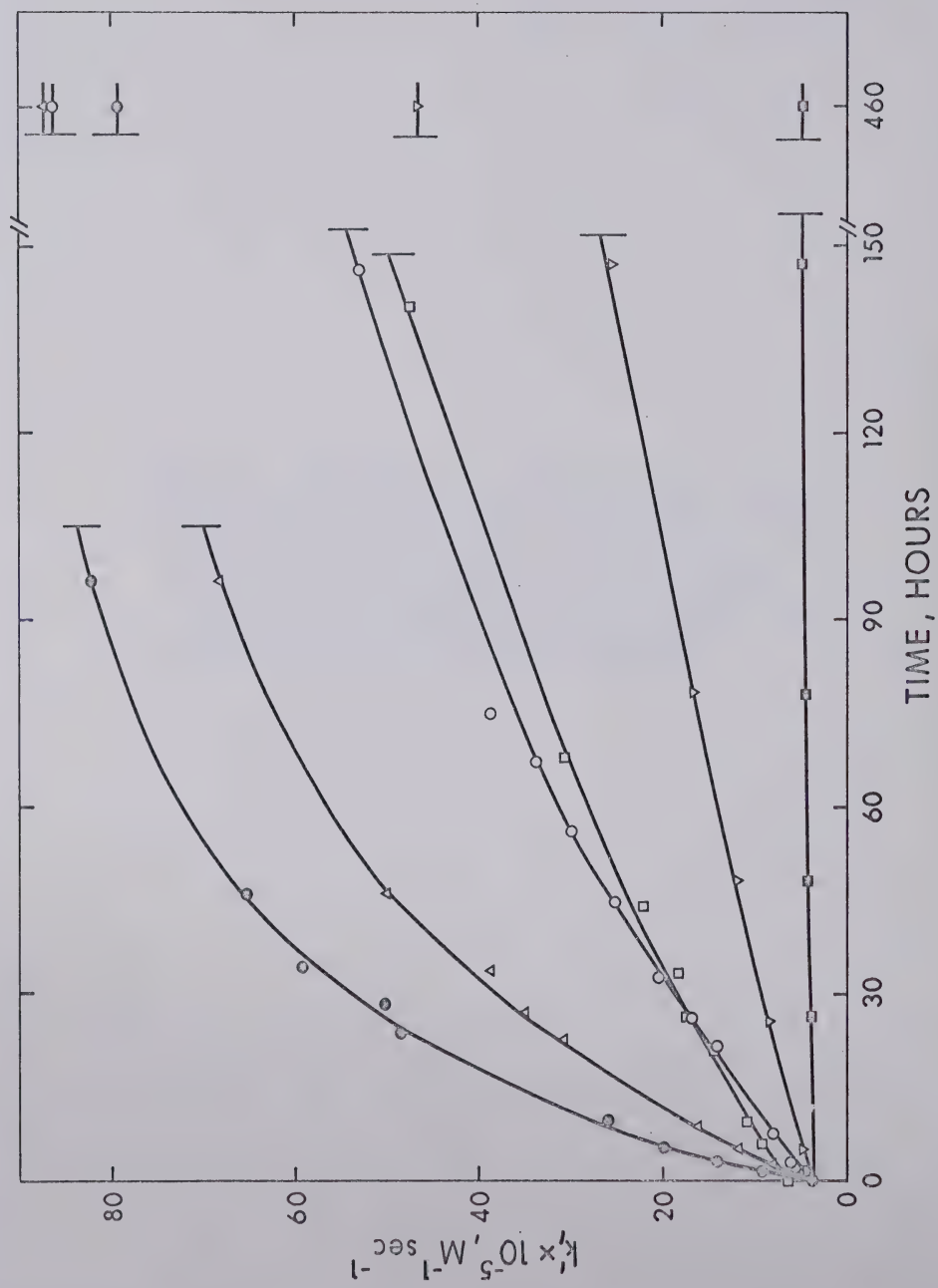


Figure 24

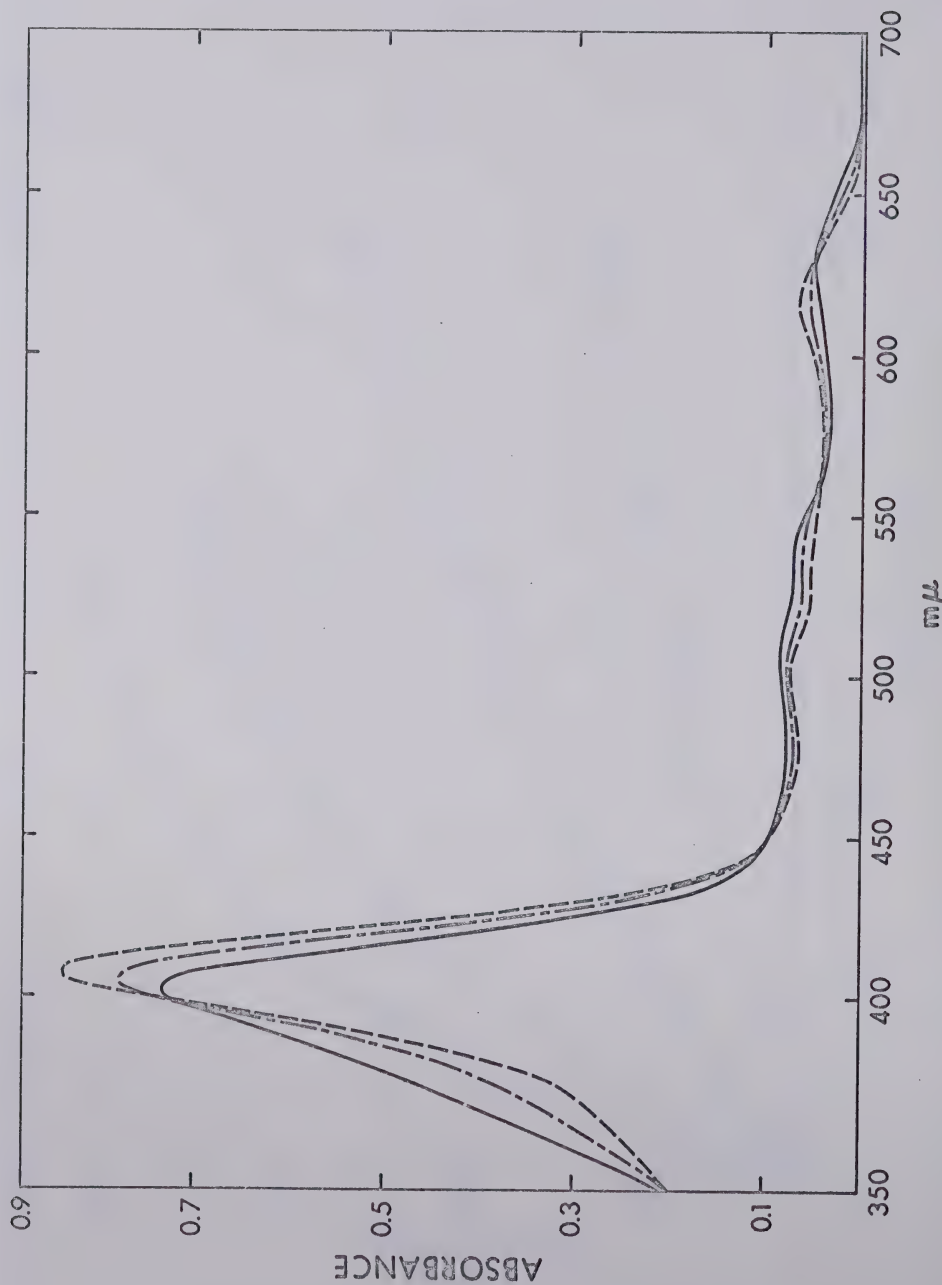


Figure 25. Regeneration of BrCN-catalase. Effect of formate on the absorption spectrum of BrCN-catalase. Spectrum of inhibited enzyme right after mixing (—), after 66 hours (---), after 214 hours (-.-.-) of incubation. 0.1 M formate, pH 4.8, 25°, 7.1 μ M catalase.

TABLE XXXII

Rate of Regeneration of BrCN-Catalase by
Formate, Acetate and Fluoride

Exp. No.	[Buffer], M	[Free Acid], mM	$k \times 10^6, \text{sec.}^{-1}$	$k \times 10^5, \text{M}^{-1} \text{sec.}^{-1}$
1	Formate, 0.3	43	8.5	20
2	-- " --, 0.2	28	4.2	15
3	-- " --, 0.1	14	1.5	11
4	Acetate, 0.2	130	0.67	0.5
5	Fluoride, 0.095	4	1.1	28

39 μM catalase, pH 4.5, 25°.

Rate constants were calculated from data given in Fig. 24.

from BrCN-modified catalase

In trying to interpret these results, four possibilities came to mind:

- $$\text{Pr} - \text{Fe} \begin{array}{c} \text{---} \text{H} \text{---} \text{X} \\ \text{---} \text{Y} \end{array} - \text{CN} \xrightarrow{\text{H}_2\text{O}} \text{Pr} - \text{Fe} - \text{Y} \quad \text{HX} + \text{CNO}^- + \text{H}^+$$

$$\begin{array}{ccc} \text{Pr} - \text{Fe} & \text{XCN} + \text{HCO}_2\text{H} & \text{Pr} - \text{Fe} \quad \text{XH} + \text{CN}^- + \text{CO}_2 + \text{H}^+ \\ \downarrow & & \downarrow \\ & & \text{HCO}_2\text{H} \\ & & \downarrow \text{catalase} - \text{HCO}_2\text{H} \end{array}$$

- $$\text{Pr} - \text{Fe} - \text{Y} \begin{array}{c} \text{H} \\ | \\ \text{O} \end{array} \begin{array}{c} \text{CN} \\ | \\ \text{X} \end{array} \quad \text{Y} = \text{H}-\text{CO}_2^-$$

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TABLE XXXIII

Effect of Ethanol and Formate on the Catalatic Activity of BrCN-Catalase

Exp. No.	Conditions of Incubation	[Catalase], μM	$k_1 \times 10^{-5}, \text{M}^{-1} \text{sec.}^{-1}$ after Incubation of						
			0 day	1.1 days	2.8 days	5.8 days	10.9 days	25.0 days	
1	0.1 M formate, 25 mM phosphate	39	6.8	7.7	6.6	8.8	-	-	
2	1 M ethanol, 8 mM phosphate	64	3.8	-	-	-	4.2	4.8	
3	2 M ethanol, 8 mM phosphate	64	3.8	-	-	-	3.9	4.5	
4	3 M ethanol, 8 mM phosphate	64	3.8	-	-	-	4.0	4.4	

pH 7.0, 25°.

Catalatic activities were determined in 5 mM phosphate, pH 7.0, at 25°, after appropriate dilutions.

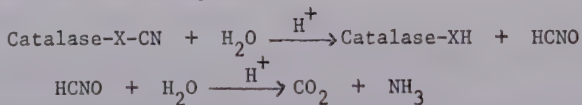
Proposal 2 was discounted, following failure to detect cyanide as a reaction product (49) and because other potential reducing agents, e.g. ethanol (Table XXXIII) were ineffective in catalase reactivation. Similarly, the stability of cyanated catalase towards formate at pH 7 argued against its role as a general base catalyst (proposal 3) (Table XXXIII). Of the remaining alternatives, the general acid catalysis was initially preferred. In accord with this concept, it was found that acetic acid and hydrofluoric acid (Fig. 24 and Table XXXII) also accelerate activation of catalase, albeit not as effectively as formate. Against the acceptance of this mechanism is the breakdown of expected linear relationship between initial velocity of reactivation and formate concentration. Instead, the initial rate is proportional to the square of total formate.

Perhaps, concurrently with the general acid function, a second molecule of formate participates in the reaction process either as a general base or as a nucleophile. Equally, the dependence on the square of HCO_2H concentration could be coincidental; for example, the ion strength was not maintained constant. Finally, it must be also admitted that no information is available on the conformation of protein under the conditions of enzyme reactivation. If it changes with increasing concentration of formate, possibly due to counter ion binding, then it may well be that XCN becomes more susceptible to hydrolytic cleavage. Curiously enough, a similar behaviour is not observed in the absence of heme binding ligands (e.g. phosphate at pH 3.5 to 5.5). Inexorably one is led to conclude that interaction of formate with ferriprotoporphyrin in cyanated catalase is an initial step in the reactivation process; a step which could be followed by some protein reorganization prior to scission of the 'CN' group.

Product of acid treatment of Br¹⁴CN-catalase

It was previously mentioned (Ch. III, p. 52) that upon acid denaturation of Br¹⁴CN-catalase the radioactivity was lost from the protein. This finding is exploited here to establish the nature of product(s) released after acid denaturation. To do so, Br¹⁴CN-catalase, prepared by using stoichiometric amounts of Br¹⁴CN, was denatured at pH 1.7 and the volatile products of hydrolysis were trapped in 0.1 M NaOH. The interconnected reaction vessels - one containing the denatured Br¹⁴CN-catalase and the other NaOH - were sealed to prevent any losses of volatile materials by evaporation. After 18 hours of incubation at 25°, the original amount or radioactivity in Br¹⁴CN-catalase, 812,000 dpm, was found to be distributed between the two solutions in the following manner: the catalase solution contained 150,000 dpm, while there was 672,000 dpm in the NaOH solution. Thus, about 83% of the ¹⁴C was trapped in the hydroxide solution.

Addition of barium chloride to the NaOH solution (final concentration ~0.02 M BaCl₂) resulted in the precipitation of almost all of the radioactivity, only 24,000 dpm was found in the supernatant. This finding indicated that the ¹⁴C released from Br¹⁴CN-catalase was trapped in the hydroxide solution as carbon dioxide. Quite likely, CO₂ is not formed directly from Br¹⁴CN-catalase but originates from the decomposition of cyanic acid:



The retention of 10 to 20% ^{14}C in the denatured protein indicates that upon acid denaturation, beside the release of ^{14}C into the medium, an intramolecular transfer of "CN" could occur giving a stable derivative or alternately the rearrangement of the modified residue into a stable form could take place. In acid medium, barring the participation of sulfhydryls, the chemistry of such a rearrangement is not immediately apparent. Under alkaline conditions, the "transfer mechanism" is more acceptable and is seemingly implied in the properties of peptides which were obtained on pronase-mediated hydrolysis of inhibited catalase (see next section).

Pronase digestion of Br^{14}CN -catalase

The ultimate proof of enzyme inhibition by the modification of a functional group in the active center, or generally, of specificity of a chemical compound towards a certain amino acid residue is the isolation and identification of the modified amino acid. This was the aim of these studies, and the stability of the BrCN -modified catalase derivative made the proposition look good. It was thought that tryptic hydrolysis of the inhibited enzyme would yield a peptide containing the modified amino acid residue; the isolation of this peptide and the identification of the modified amino acid residue could, then, be easily achieved. However, any hope of success was soon dispelled as it was found that upon denaturation the radioactivity was lost from Br^{14}CN -catalase. It has been shown earlier (see page 52) that the label was lost when Br^{14}CN -catalase

was denatured under acidic conditions. Similar results have been obtained when the radioactive protein was denatured in an organic solvent, dimethyl sulfoxide, or by heating at 90° for 5 minutes. Thus, it seemed that as soon as the modified residue was exposed to the medium by denaturation the modification was reversed and the ^{14}C was released. Since undenatured catalase is resistant to the action of trypsin, the isolation of tryptic peptides had to be abandoned.

Some hope of success was kept alive by the finding that there was only limited 10 to 40% loss of ^{14}C when Br^{14}CN -catalase was digested by pronase at pH 8.4. Spurred by this observation the following experiments were performed. Br^{14}CN -catalase, containing one equivalent of ^{14}C , was digested by pronase (catalase/pronase = 28, w/w) at pH 8.4 and 35° for three hours. Ultrafiltration (Diaflow ultrafilter, UM-1, 10,000 MW cutoff) of the hydrolyzate gave a solution containing 87% of the original radioactivity. Since peptides containing more than 40 to 50 amino acid residues are not expected to pass through the filter rapidly, the protein was quite thoroughly digested. An aliquot of the filtrate containing 4,200 dpm, was then spotted on Whatman 3 MM paper and subjected to flat-bed electrophoresis at pH 8.2 using 0.1 M borate as supporting buffer (for experimental details see Chapter II).

The distribution of ^{14}C among the peptides on the paper strip was determined on the Nuclear Chicago strip counter and radioactivity was quantitated following calibration with standards whose properties were, in turn, evaluated by liquid scintillation counting method. In

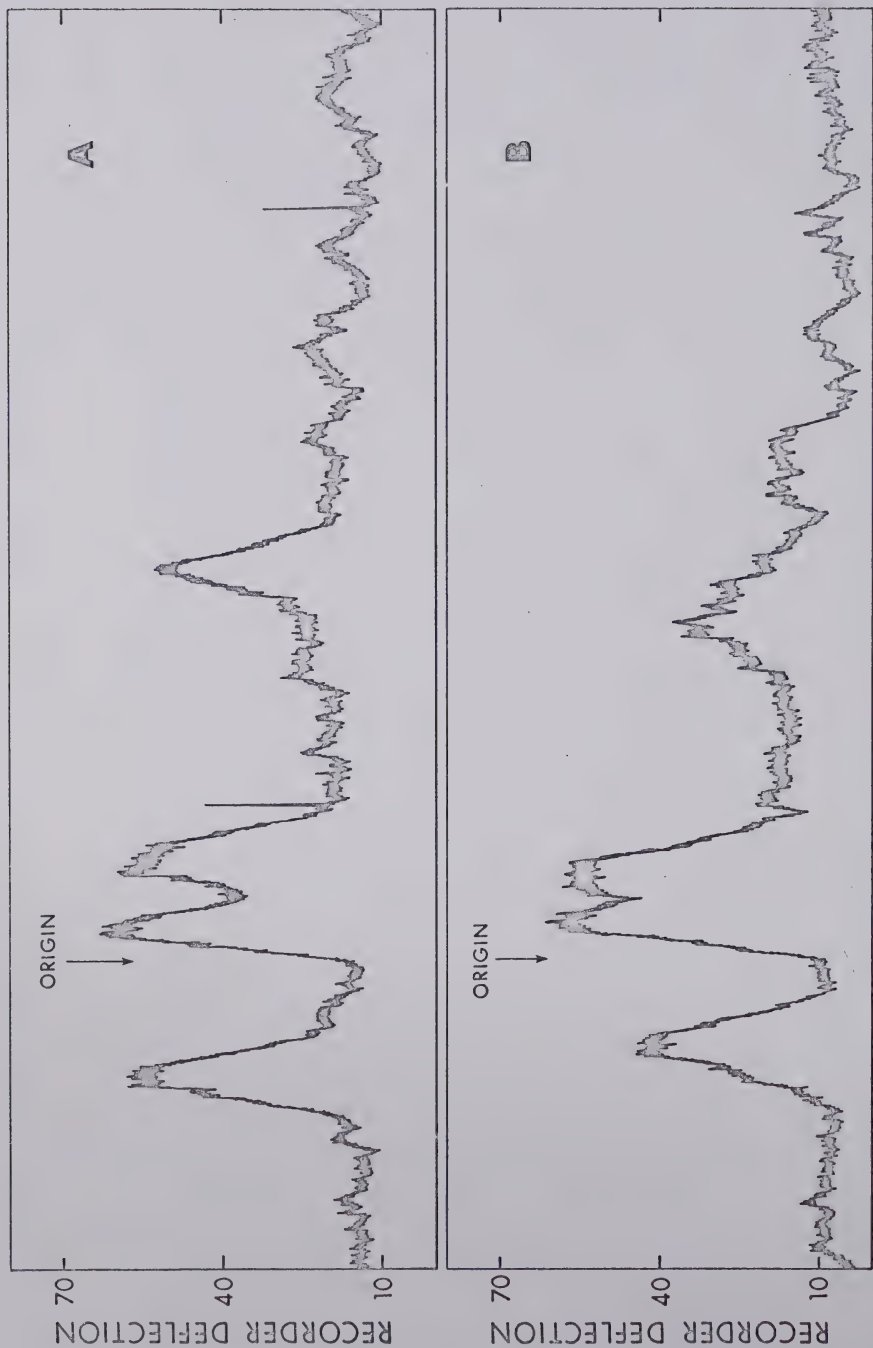


Figure 26. Radioactivity patterns of Br^{14}CN -catalase peptides after electrophoresis at pH 6.5.
A: filtrate of pronase treated Br^{14}CN -catalase. B: the same filtrate after acid treatment.
See text for experimental details.

TABLE XXXIV

Effect of Acidification on Pronase-Treated BrCN-Catalase

	Spotted on Paper Strip d.p.m.	Found on Paper Strip d.p.m.
Filtrate of pronase-treated Br ¹⁴ CN-catalase	4,200	~3,500
Acidified filtrate of pronase- treated Br ¹⁴ CN-catalase	4,200	~3,600

For experimental detail see text.

this manner, it proved possible to calculate that, after electrophoresis, about 65% of the radioactivity present initially in $\text{Br}^{14}\text{CN-catalase}$ was retained in the peptides on the paper strip.

The radioactivity pattern shown in Fig. 26A suggested that the label was attached to at least four different peptides. This was somewhat disturbing but it could have been explained by incomplete or differential hydrolysis by pronase. However, before considering improvements of the proteolysis procedure, one crucial test was essential - namely, to show that radioactivity is lost after acidification of the pronase hydrolyzate. The loss of label upon acid treatment could be interpreted as an indication that the modified residue was still in the same chemical form as it is when $\text{Br}^{14}\text{CN-catalase}$ is denatured under acidic conditions. Consequently, a portion of the same filtrate obtained after pronase hydrolysis was acidified to pH 2, incubated for three hours at room temperature, and then the pH was readjusted to 8.4. Electrophoresis, under the above described conditions, gave essentially the same peptide and radioactivity patterns and, what is more important, no loss of radioactivity occurred (see Fig. 26B and Table XXXIV).

The retention of radioactivity in the pronase hydrolyzate, even after acid treatment, strongly suggests that during pronase hydrolysis the transfer of ^{14}C takes place, from the originally modified site to another amino acid residue that is probably brought into the proper position by the structural changes, which take place during the pronase digestion. In this manner the transfer of the ^{14}C could take place giving rise to possible derivatives that are stable

under the slightly alkaline and the subsequent acidic conditions. The appearance of the label in more than one peptide can be explained by the transfer of the ^{14}C to more than one amino acid residue that are situated at different positions along the polypeptide chain, even if it is supposed that uniform digestion products are obtained by the pronase treatment.

Looking at the primary aim of these studies it became evident at this stage that neither the kinetic approaches nor the use of available analytical techniques will permit the definite identification of the amino acid residue that is modified when catalase is inhibited by BrCN . Under these circumstances, it seemed worthwhile to compare the properties of BrCN -modified catalase with those of a corresponding compound formed on treating metmyoglobin with cyanogen bromide.

CHAPTER V

Modification of Metmyoglobin by Cyanogen Bromide

Relationship of structure and function in myoglobin

Myoglobin was one of the first proteins whose three-dimensional structures were established by x-ray crystallography (79,147,148). The results of these investigations confirmed many of the structural features which had been deduced previously from chemical and physico-chemical studies, and in addition revealed many new and unsuspected structural details.

The molecule of myoglobin is very compact, with virtually no access to its interior for solvent molecules. The heme group is situated in a pocket of the polypeptide chain, but near the surface; the vinyl side groups of the heme extend into the hydrophobic interior, while the propionic acid groups form a part of the polar surface of the molecule.

The structural features of myoglobin, which are particularly relevant to this study, are the ones defining the immediate environment of the prosthetic group. The following amino acid residues surround the heme (164a,164b): on the proximal side; two leucines (F4, G5^{*}), phenylalanine (H 14), isoleucine (H 18), and histidine (F 8); on the distal side, two leucines (B 10, B 13), two phenylalanines (B 14, CD 1), isoleucine (G 8), threonine (C 4), and histidine (E 7). Histidine F 8 is coordinated to the heme at the fifth position (proximal side), while histidine E 7 is hydrogen

*The system of labelling the amino acid residues is given in ref (148).

bonded to the water molecule coordinated to the heme at the sixth position in metmyoglobin (distal side). Threonine C 4 was shown to be hydrogen bonded to a main chain carbonyl. Therefore, it is apparent that on the distal side of the heme, where ligands are bound, only histidine E 7 and threonine C 4 could, by the nature of their side-chain functional groups, possibly participate in the binding of ligands by this protein.

Since the physiological function of myoglobin and the properties of oxymyoglobin were described, the main objective of the many studies on myoglobin has been to provide a satisfactory explanation for the unusual stability of its oxygenated derivative.

Although x-ray crystallographic studies resulted in the elucidation of the detailed three-dimensional structure of myoglobin and some of its derivatives, similar studies on oxymyoglobin have been greatly hampered by its instability under the conditions necessary for the application of this method. Other lines of investigations, which have provided information about the properties of myoglobin, have been concerned with model systems, other oxyferrohemo proteins, and theoretical studies.

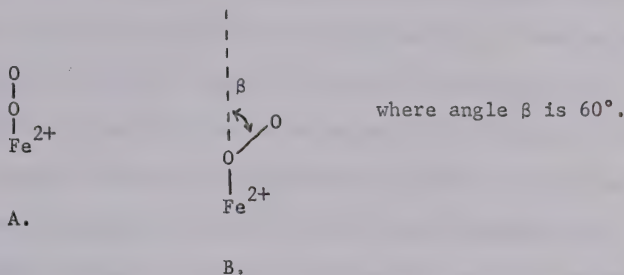
Recent studies with iron (II) porphyrins have explored the nature of binding involved in the CO and O₂ derivatives of these compounds. The effect of changing the medium and the substituents at positions 2 and 4 of the porphyrin ring on the reactions of these compounds with CO and O₂, and the stability of the complexes formed have been examined (164c, 164d, 165a). When pyridine solutions of iron (II) deuteroporphyrin IX derivatives were diluted with various solvents,

such as ethanol, acetone, water, and acetic acid, the oxygen present in these solutions displaced one of the pyridines coordinated to the iron. This oxygenation reaction was facilitated as long as the diluent was protic in character, because pyridine is less effective as a ligand when protonation of its nitrogen takes place. The formation of the oxygenated derivatives was followed by oxidation of the iron to its ferric state, although no detailed description of these reactions was given. The most significant aspect of these studies was the effect of the substituents in positions 2 and 4 of the porphyrin ring on the formation of the oxygenated derivative. The more electron-withdrawing the substituents were the slower was the formation of the oxygenated complex. Conversely, CO was more firmly bound by iron (II) porphyrin when these substituents were more electron-donating in character (78,80,141).

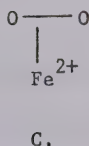
These studies were extended to various myoglobin and hemoglobin preparations, which had been obtained by reconstitution of the apoprotein with different iron-porphyrin derivatives. It was shown that deuterohemoglobin bound oxygen two times and mesohemoglobin six times more strongly than reconstituted protohemoglobin (156,157). Similarly, deuteromyoglobin had three times as great an affinity for oxygen as protomyoglobin (158). Thus, oxygen binding increased as the 2,4-substituents were changed from vinyl (proto) to hydrogen. (deutero) and to ethyl groups (meso).

These results appear to indicate that the strength of CO and O₂ binding in the case of hemoglobin and myoglobin derives from the ability of the iron (II) to form a double bond with these pi-

acceptor ligands; the iron (II) serves as an acceptor, to form a σ -bond, and at the same time donates electrons to the ligand to form a π -bond. Such bonding is consistent with the models proposed for oxygen binding by Pauling (59) and Griffith (58). Pauling proposed the following models for the binding of oxygen by hemoglobin and myoglobin:



while Griffith suggested the following structure:



(In all three models Fe^{2+} represents iron (II) protoporphyrin IX). Calculations based on these models indicated that if an oxygen molecule were bound perpendicularly to the heme plane (model A) iron (II) would be readily oxidized to iron (III), whereas if the oxygen molecule were parallel to the heme plane, (model C), the iron (II) would be quite stable in its lower oxidation state. A model compound, chlorocarbonyl - bis (triphenylphosphine) iridium, has recently been shown to be a reversible oxygen carrier, and in its oxygenated form

the oxygen molecule was found to be bonded to the metal in the manner proposed by Griffith (61,159).

Recently, the distal and proximal histidines of myoglobin (and hemoglobin) have been considered to have a role in the binding of oxygen (164e). It has been suggested that the strong pi-donation by iron could contribute a polar character to the metal-oxygen bond, and therefore oxygen binding would be stabilized by an interaction, such as hydrogen bonding, between the distal histidine and the oxygen molecule. This postulate is supported by the results of x-ray studies which show hydrogen bonding between the distal histidine and azide in metmyoglobin-azide (142). The proximal histidine could serve as a sigma-donor not only to stabilize the iron (II)-oxygen complex, but also to prevent overly strong oxygen binding, as would be required for a protein whose function is reversible oxygen binding (165a).

The stabilities of oxymyoglobin and oxyhemoglobin can be put into the proper perspective if the stabilities, or for that matter the existence, of other oxyferrohemoproteins are considered. Just recently Yamazaki (64f) and Wittenberg (160,161) and their coworkers have described conditions under which the oxyferro-derivative of horse radish peroxidase can be obtained, and showed it to be equivalent to the previously described compound III of peroxidase. Oxyferroperoxidase is quite unstable, the half time of decomposition is about 4 minutes, even under strictly controlled conditions, and it readily reverts to ferric-peroxidase in the presence of hydrogen donors and oxidizing agents (hydrogen peroxide). Reversible

dissociation of oxygen from the oxygen from the oxyferro-derivative could not be demonstrated. The absorption spectrum of oxyferroperoxidase was not affected by variation in temperature; in contrast, the absorption spectrum of oxymyoglobin showed temperature dependence. As a result the visible absorption spectra of oxyferroperoxidase, CO-ferroperoxidase, oxymyoglobin, and oxyhemoglobin were almost identical at 77°K. These facts indicate that at room temperature oxymyoglobin is an equilibrium mixture of two forms, while oxyferroperoxidase exists only in one electronic configuration. Maybe this is the explanation for the reversible oxygenation of myoglobin.

In the case of other hemoproteins, such as cytochrome c peroxidase (161), cytochrome oxidase (76,77,164g), and tryptophan pyrolase (165b), the transitory existence of the oxyferro-derivative has either been demonstrated, or at least inferred by studies of the mechanism of action of these enzymes, but obviously their stabilities do not, at all, approach that of oxyglobin.

Now the question can be asked which of the structural features make myoglobin and hemoglobin oxygen carriers, while the other hemoproteins form oxygenated derivatives which have only very limited stability. Clearly, these differences must be due to variations in the protein environment of the heme group, specifically in the amino acid residues near the fifth and sixth coordination positions. A direct, unambiguous approach to this problem would be the chemical modification of the distal and/or proximal histidine residues of myoglobin, and the examination of some of the properties of the derivative(s), such as rate of oxygenation, strength of

oxygen binding, and stability of the oxygenated form. This would enable us to evaluate the role these residues play in defining the physiological function of myoglobin.

Unfortunately, nobody has, as yet, been able to specifically modify either of the histidine residues in the vicinity of the heme in myoglobin. The difficulty of this approach probably stems from the fact that the reagents developed so far are not specific enough for histidine; that is, strictly controlled conditions are required to limit the reaction of the reagent to histidine residues only. This is the case, for example, with photochemical oxidation in the presence of methylene blue (63), or with aryl halides, such as 1-fluoro-2,4-dinitrobenzene (62). These methods could result in the modification of not only histidine, but also other amino acid residues like cysteine, methionine, tryptophan, lysine, tyrosine and so on. On the other hand, Gurd and his coworkers have shown that the reactivity of certain alkylating agents, such as bromoacetate and iodoacetamide, can be limited solely to histidine residues in myoglobin under properly controlled conditions (163 and references therein). However, they succeeded in modifying histidine E7 only when the exhaustive alkylation had caused the denaturation of the myoglobin while histidine F8 was not attacked at all (162). Thus it appears that in native myoglobin these histidine residues are sterically inaccessible to the alkylating agents or are resistant to attack because of their interaction with the heme group.

Based on the results obtained with catalase it was felt that cyanogen bromide could be a reagent of sufficient specificity, since

its reaction appears to depend on specific interaction with the prosthetic group. Therefore, it was hoped that in this manner the reagent would react, in the vicinity of the heme, with an amino acid residue which plays a role in the function of myoglobin.

The discovery of a specific inhibition of catalase led to enquiries on the susceptibility of other hemoproteins towards cyanogen bromide. A number of considerations led to the selection of myoglobin as the second hemoprotein whose interaction with cyanogen bromide has been examined. The first consideration was the ready availability of purified myoglobin that permitted experiments which could not be carried out with catalase. Secondly, the known structure of myoglobin would permit the interpretation of results in terms of basic chemistry more readily even if the direct demonstration of the modification, such as isolation of the modified residue, were not possible. In comparison with the catalase-BrCN system the investigation of the metmyoglobin-BrCN interaction appeared simpler because the number of potentially reactive groups is greatly reduced in myoglobin (molecular weight 17,800 compared to 250,000); furthermore, metmyoglobin contains no cysteine residues and thus one of the difficulties encountered with catalase is eliminated. On the other hand, the effect of BrCN-modification on the function of metmyoglobin could not be as readily assessed as in the case of catalase, mainly because of the instability of the modified metmyoglobin derivative (see page 95). This finding makes the system unsuitable for analytical work involving any but relatively fast techniques and thus precludes attempts to isolate the modified residue. In this respect, it is

significant that crystalline metmyoglobin is modified by BrCN without disrupting the crystal lattice and thus crystallographic analysis is possible.

The experimental evidence obtained in this study, as it will be described, suggests that the modification of metmyoglobin involves reaction of BrCN with the distal histidine residue. However, the instability of the modified derivative prevented the direct demonstration of the histidine modification. In addition, the available instrumentation was not suitable to examine the physiological function of the modified derivative, as it was outlined in the introduction.

Preparation of metmyoglobin solutions

Sperm whale metmyoglobin (MMb) used in this work was obtained from Mann as twice crystallized, salt-free, lyophilized powder. The stock solutions, of concentrations around 2 mM, were prepared in glass distilled water and following centrifugation of any insoluble material, the solutions were assayed by the hemochromogen method (see page 27)*. The results obtained were in excellent agreement with calculations based on molar extinction coefficients, using $\epsilon_{409} = 169 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{504} = 9.05 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, and $\epsilon_{633} = 3.65 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (144). No attempts were made to remove possible protein contaminants by further purification, mainly because preliminary experiments showed

*The hemochromogen of myoglobin has an absorption spectrum identical to that of catalase (see Fig. 6), except that the slight shoulder in the 600 to 650 mμ region is not present in the case of myoglobin.

that the reaction of BrCN with metmyoglobin can be almost quantitatively accounted for on the basis of intact hematin-Fe present. Thus, neither the main bulk of apomyoglobin nor the possible protein contaminants react with BrCN under the conditions used to characterize the BrCN-modification process.

Reaction of metmyoglobin with cyanogen bromide.

Rate of reaction. The reaction of metmyoglobin with cyanogen bromide was monitored spectrophotometrically at 410 m μ , taking advantage of the difference between the absorption spectra of native and BrCN-modified metmyoglobins (see Figs. 30 and 31). The reaction is characterized by a second order rate constant of $31.5 \pm 2.0 \text{ M}^{-1} \text{ sec}^{-1}$, in 0.1 M phosphate, pH 7, 25°; this average value includes a large number of determinations (about 35) under a wide variety of conditions. In one set of experiments excess metmyoglobin, 5 to 10 fold more than BrCN was used, while in a number of others the concentrations of BrCN exceeded those of metmyoglobin by a factor of 10 to 100. In a few experiments equimolar amounts of BrCN and metmyoglobin were allowed to react. Since all the rate constants obtained under these widely varying conditions came nearly to same value, it is evident that the rate of the modification reaction depends on the concentrations of both metmyoglobin and BrCN. In addition, under the conditions used no intermediate could be detected by the spectrophotometric method utilized.

Stoichiometry of the BrCN-Metmyoglobin Reaction

Exp. No.	Total [MMb], μM	"Free" [MMb] ^a , μM	[BrCN] μM	Expected ^b ΔA_{540}	Observed ΔA_{540}	Obs. ΔA_{540}	
						Exp.	ΔA_{540}
1	431	431	89.6	0.309	0.258	0.835	
"	429	429	89.3	0.307	0.227	0.739	
2	434	434	54.4	0.187	0.174	0.931	
"	433	~380	54.3	0.187	0.173	0.926	
"	431	~430	54.1	0.186	0.178	0.952	
"	429	~375	53.9	0.186	0.183	0.984	
"	410	~390	390	0.414	0.377	0.910	

0.1 M phosphate, pH 7.0, 25°.

Between BrCN additions BrCN-MMb was partially or completely regenerated.

^a Includes native MMb and regenerated MMb.

^b Expected absorbance change on the basis of added BrCN.

^c Corresponds to the ratio $[\text{BrCN-MMb}](\text{formed}) / [\text{BrCN}](\text{added})$.

The pH-dependence of the reaction rate. These experiments were carried out to see if an ionizable group, whose pK falls within the pH range investigated, is involved in the modification reaction. The rate of the metmyoglobin-BrCN was measured under the conditions indicated in Fig. 27 by following the formation of the modified protein spectrophotometrically. The results of the rate measurements in the pH range of 4.5 to 9.2 are given in Fig. 27; although there are significant variations, especially in the pH region of 4.5 to 6.5, there is no definite trend evident that would indicate the involvement of an ionizable group in the reaction. This leaves three possible explanations:

- a) the state of ionization of the group does not change in the pH range studied.
- b) a non-ionizable group is involved
- c) the proton exchange does not take place in the rate determining step.

It will be shown in Chapter VI (see page 99) that the last alternative is the most likely one, mainly because the modification of a histidine residue is strongly suggested by experimental evidence to be presented in a subsequent section of this Chapter. However, the discussion leading to the selection of the third alternative will be given in Chapter VI to provide an explanation for similar observations in the catalase-BrCN system.

Stoichiometry of the BrCN-metmyoglobin reaction. The experiments described here were undertaken to show how many BrCN molecules are required to modify one metmyoglobin molecule. The experiments were carried out under the conditions given in Table XXXV, by following the formation of the modified metmyoglobin spectrophotometrically at 540 m μ . This wavelength was selected to obtain a large absorbance change to monitor the reaction; on the other hand, lower wavelengths could not be used because of the high absorbance of the protein (for spectra, see Fig. 31).

In the experiments outlined in Table XXXV, less than stoichiometric amounts of BrCN ([MMb]/[BrCN] ratio ranged from ~ 4 to ~ 10) were added to the metmyoglobin solutions. In this manner the modification reaction was fast, $t_{1/2} = \sim 1$ min, compared to the regeneration process, $t_{1/2} = \sim 1$ hour. The regeneration was partly or wholly completed before the next addition of BrCN. This is indicated by the figures in the third column of Table XXXV, when the value under "Free" [MMb] is smaller than the corresponding one under Total [MMb], then the regeneration was not complete. As the results indicate the modified metmyoglobin present did not influence the stoichiometry of the reaction of the remaining metmyoglobin.

The expected absorbance changes were calculated on the basis of the added BrCN by assuming that all the BrCN reacted to give modified metmyoglobin, and by using a value of $3.95 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for $\Delta\epsilon_{540}$. The values listed in the last column indicate the number of moles BrCN-modified metmyoglobin formed per mole of BrCN added; they were calculated as indicated by the expression at top of the column.

Heme Removal from Br¹⁴CN-Metmyoglobin

Exp. No.	Removed at ^a	Amount of MMb, nmoles	Amount of ¹⁴ C, d.p.m.	Method of Acidification	Amount of ¹⁴ C Found in	
					Aqueous phase	Organic phase
					d.p.m. %	d.p.m. %
1	12 min.	39.4	154,000	HCl, pH 1.8	104,000 68	36,000 24
"	15 min.	--"	-- "	0.1 M F ⁻	127,000 82	20,000 13
2	9 min.	--"	-- "	-- "	124,000 81	30,000 20
"	16 min.	--"	-- "	-- "	131,000 85	18,000 12
3	10 min.	168.6	690,000	0.25 M F ⁻	650,000 94	52,000 8
4	-- "	--"	-- "	-- "	648,000 94	53,000 8
5	-- "	--"	-- "	-- "	640,000 93	54,000 8

Br¹⁴CN-MMb was obtained by adding about equimolar quantity of Br¹⁴CN to MMb;

0.1 M phosphate, pH 7.0, 25°; MMb concentrations ranged from 390 to 560 μ M.

For inhibition reaction: t-1/2 = 1 to 1.5 min.

^a Time after the addition of Br¹⁴CN to MMb.

The good correspondence between the amount of BrCN added and the amounts BrCN-metmyoglobin formed, as shown in Table XXXV, clearly indicate that the modification of metmyoglobin by BrCN is the result of a specific reaction between the reagent and a certain locus of the hemoprotein molecule. Under favorable conditions, as in Exp. 2 in Table XXXV, not more than 10% of the reagent is used up in unspecific side-reaction(s).

Heme removal from Br¹⁴CN-metmyoglobin. The aim of the experiments detailed in this section was to show which part, heme or protein, of the metmyoglobin molecule is modified by BrCN. The experimental approach utilized the established procedure of Teale (128), and a recently modified version of it (145), to separate the heme and apoprotein parts of the metmyoglobin molecule. 2-butanone was used to extract heme moiety from the acid denatured metmyoglobin.

Br¹⁴CN-metmyoglobin was prepared by the addition of equimolar amounts of Br¹⁴CN to metmyoglobin (concentrations ranged from 390 to 560 μ M) in 0.1 M phosphate, pH 7, 25°. Under these conditions the half time of the reaction is 1 to 1.5 minutes. Nine to 16 minutes after the Br¹⁴CN addition, aliquots were removed and treated in the following manner (refer to TABLE XXXVI): In Exp. 1, cold glass distilled water was added to the first aliquot and then the pH was lowered to 1.8 by adding a predetermined amount of concentrated HCl; in all other cases the proper volumes of cold 1.0 M fluoride, pH 5.2 and cold glass distilled water were added to the aliquots to obtain the fluoride concentrations indicated in Table XXXVI (final

fluoride concentrations after the addition of 2-butane). The third and fourth columns in Table XXXVI indicate the amounts of metmyoglobin and radioactivity present in the aliquots. The addition of fluoride was quickly followed by the addition of equal volume of cold 2-butanone. The solution was mixed by inversion for 30 sec and then the phases were allowed to separate (2-3 minutes). The aqueous and organic phases were removed into separate tubes and the radioactivity was determined in both by liquid scintillation counting. The results of these determinations are also given in Table XXXVI.

The data obtained clearly indicates that the major portion of the label remains associated with the protein portion of Br^{14}CN -metmyoglobin after the removal of heme. Under the more acidic conditions, at pH 1.8, the hydrolysis of Br^{14}CN -metmyoglobin is faster (see page 97), resulting in the release of more ^{14}C . If it is assumed that HO^{14}CN is released as the result of the acid hydrolysis (as it will be shown later, see page 97), then the uncharged cyanic acid molecule (pK_a of cyanic acid is ~ 3.5) will probably be soluble to a certain extent in the organic phase. Thus, more radioactivity appears in the organic phase in comparison with the results obtained at the higher pH value.

The difference observed between the two sets of results obtained at 0.1 and 0.25 M fluoride concentrations could be taken as an indication that the reversion of BrCN -metmyoglobin (to the native protein) by hydrolysis is achieved by the attack of a water molecule that is activated by its coordination to the hematin-Fe. In this manner at the higher concentration of fluoride the release of ^{14}C by

The Release of Bromide in the BrCN-Metmyoglobin Reaction

Experiment 1			Experiments 2 and 3			
[Br ⁻], μM	-log [Br ⁻]	Relative Recorder Deflection, Divisions	[BrCN], μM	[Br ⁻], μM	-log [Br ⁻]	Relative Recorder Deflection, Divisions
						<u>Exp. 2</u> <u>Exp. 3</u>
33.1	4.480	0	-	16.6	4.780	0 0
66.1	4.180	25	-	33.1	4.480	20 19
98.8	4.005	42	34.1 ^a	67.1 ^a	4.173	43 45
131.2	3.882	54	-	83.6	4.078	53 54
163.5	3.786	64	-	116.3	3.934	65 67
195.6	3.709	73	-	149.4	3.826	76 77

Glass distilled water, 197 μM MMb, pH 6.5, 25°.

^a It was assumed that the added BrCN was quantitatively converted to Br⁻ in the course of the BrCN-MMb reaction.

The above data was plotted in Fig. 28.

hydrolysis is slower, because the removal of the heme is probably faster in the presence of the higher concentration of fluoride (145), and therefore a smaller amount of the label could appear in the organic phase.

From the results obtained in these experiments it is evident that the ^{14}C of the Br^{14}CN used to modify metmyoglobin is associated with the protein portion of molecule. It appears very unlikely that the label could be transferred almost quantitatively to the protein portion during the separation procedure if the ^{14}C was associated with the heme initially.

Release of bromide during the modification of metmyoglobin by BrCN .

It was also of interest to determine what happens to the bromine portion of the BrCN molecule during the modification. If the bromine were retained in BrCN -metmyoglobin, then addition to a double bond or the formation of an internal salt could be envisaged. On the other hand, the release of bromide ion during the modification would indicate a substitution reaction, that would probably be accompanied by the release of a proton.

The experiments were carried out by measuring the potential changes that occurred when standardized ammonium bromide was added to the solution. An Orion bromide electrode was utilized, the reference electrode being a radiometer saturated calomel one. The sensitivity of the recorder was set at 5 mV per hundred divisions. In Exp. 1 (see Table XXXVII) the indicated amounts of ammonium bromide were added to the metmyoglobin solution and the recorder deflections

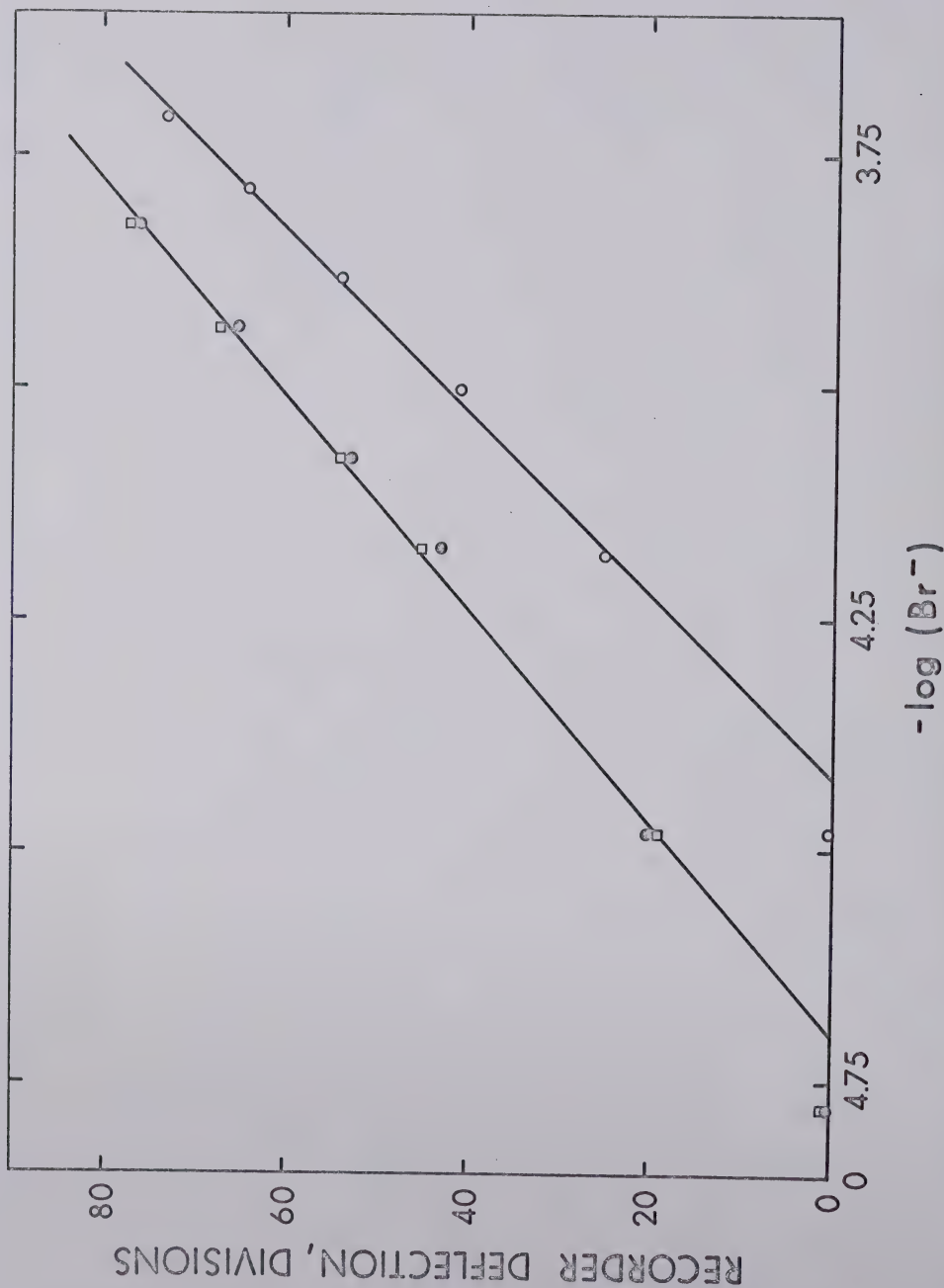


Figure 28. The release of bromide in the metmyoglobin-BrCN reaction. Data from Table XXXVII.
Exp. 1 (o), Exp. 2 (●) and Exp. 3 (□).

obtained are given in Table XXXVII. In Exp's. 2 and 3 the indicated amount of BrCN was added to the same metmyoglobin solution between additions of ammonium bromide. The change in potential was followed for about 15 minutes after the BrCN addition (under the conditions used $t-1/2$ of the MMb-BrCN reaction is about 2 minutes), before the next addition of ammonium bromide was made. In the calculation of bromide concentrations it was assumed that the BrCN added was quantitatively converted to bromide as the result of the modification of metmyoglobin by the reagent (see Table XXXVII).

The data listed in Table XXXVII were plotted in Fig. 28. The straight lines obtained in all three experiments show that there is a fairly good relationship between the logarithm of bromide ion concentrations and the observed potential changes. Furthermore, the linear relationship also indicates that the assumption made in the calculation of bromide concentrations in Exp's. 2 and 3 was correct; that is, in the course of the MMb-BrCN reaction bromide ion is liberated from BrCN in quantitative amounts. This finding suggests, as it was indicated above, that the modification of metmyoglobin by BrCN should be accompanied by the liberation of hydrogen ion.

Before going on to the next topic it has to be mentioned here that a rough estimate of the rate of bromide ion liberation was made. (More reliable data could not be obtained because of technical difficulties). The second order rate constant for the liberation of bromide ion was found to be $37 \pm 6 \text{ M}^{-1} \text{ sec}^{-1}$ which is in reasonable agreement with the rate constant for BrCN-metmyoglobin formation (see page 83).

TABLE XXXVIII

Proton Release in the BrCN-Metmyoglobin Reaction

Exp. No.	[MMb], μM	[BrCN], μM	$[\text{H}^+] / [\text{BrCN-MMb}]^a$	pH Range Used
1	935	950	0.78	6.94 - 6.68
2	970	197	1.01	6.93 - 6.85
"	~770	195	1.06	6.85 - 6.76
3	980	205	1.02	6.93 - 6.85
"	~780	205	1.04	6.85 - 6.77

0.05 M KCl, 25°.

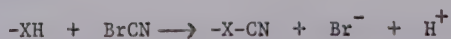
Changes in $[\text{H}^+]$ were calculated by using calibration data obtained with standardized HCl and NaOH solutions in a similar MMb solution.

^a Amount of BrCN-MMb formed was assumed to correspond to the amount of BrCN added.

Proton release in the BrCN-metmyoglobin reaction. The results obtained in the previous section indicated the possibility that hydrogen ion could be liberated in the metmyoglobin-BrCN reaction. The experiments described here were attempted to examine this aspect of metmyoglobin modification by BrCN.

The modification of metmyoglobin by BrCN was carried out under the conditions indicated in Table XXXVIII and the pH changes were monitored by using a recorder. The pH changes were calibrated by standardization with dilute HCl and NaOH in similar metmyoglobin solutions over the entire pH range utilized. In Exp. 1 (see Table XXXVIII) the endpoint of the reaction could not be determined with reasonable confidence because of the continued, but slower proton liberation which must be associated with the regeneration process. The conditions used in Exp's 2 and 3 appeared to be much better in this respect; in these experiments two portions of BrCN were added, the second one after the first reaction was completed (8 - 10 minutes after the first addition). The ratio $[H^+]$ released/[BrCN-MMb] formed was calculated by assuming that all the metmyoglobin was converted to BrCN-metmyoglobin, or all the BrCN reacted to give modified metmyoglobin, whichever the case was.

The results clearly indicate that one hydrogen ion is liberated for each metmyoglobin modified by BrCN. This suggests that the modification reaction could take place in the following manner:



where -XH is a protein functional group and could very well be the imidazole group of a histidine residue.

The reliability of rate measurements was also poor in this case; the rate constant for the liberation of hydrogen ion was found to be $30 \pm 6 \text{ M}^{-1} \text{ sec}^{-1}$ in reasonable agreement with that obtained for the formation of the modified derivative.

Properties of native and BrCN-modified metmyoglobins

Sedimentation constant and circular dichroism. The measurement of some physical properties of native and modified metmyoglobins was carried out to determine if the modification by BrCN is accompanied by changes in the structure of the protein. Since these measurements take a relatively long time, 1 to 2 hours, special precautions were observed to ensure that the metmyoglobin was in its BrCN-modified form while the measurements were taken. This was achieved by the addition of at least 10-fold excess of BrCN to obtain the modified metmyoglobin; under these conditions, reversion of BrCN-metmyoglobin does not occur for several hours. This was verified by looking at the absorption spectra of the BrCN-metmyoglobin solutions after the completion of the ultracentrifuge and CD experiments (about 3 hours after BrCN addition). The absorbance readings taken in the Soret region indicated that not less than 95% of metmyoglobin present was in its modified form at the time of the absorbance measurements.

For the ultracentrifuge run, BrCN-metmyoglobin was prepared by adding 1.78 mM BrCN (in acetonitrile) to 175 μM metmyoglobin in 0.1 M

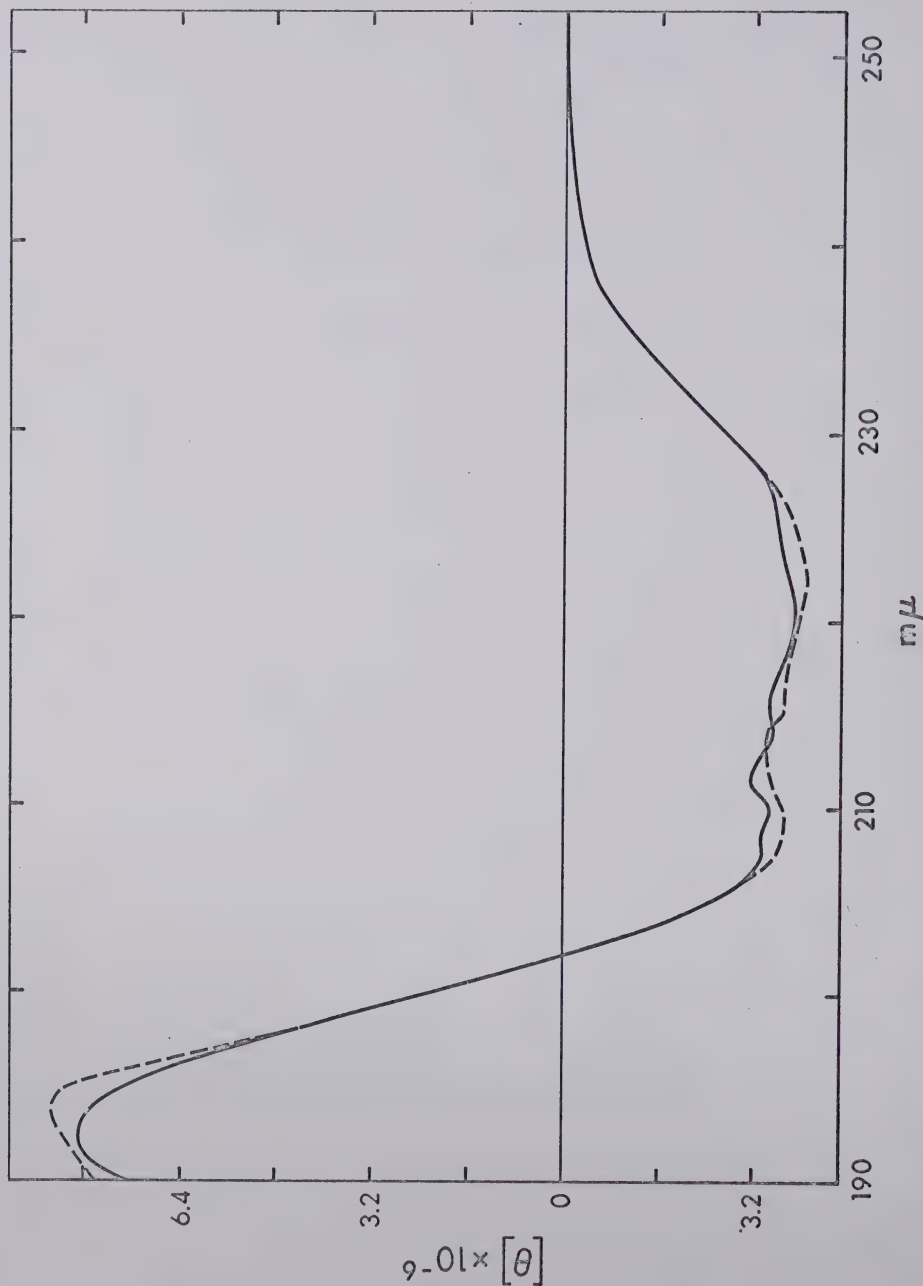


Figure 29. Ultraviolet circular dichroism spectra of metmyoglobin (—) and BrCN-metmyoglobin (---). Experimental details as described for catalase (see page). Molar ellipticities, $[\theta]$, were calculated in terms of heme concentrations, and have the dimensions of degrees·cm² per decimole.

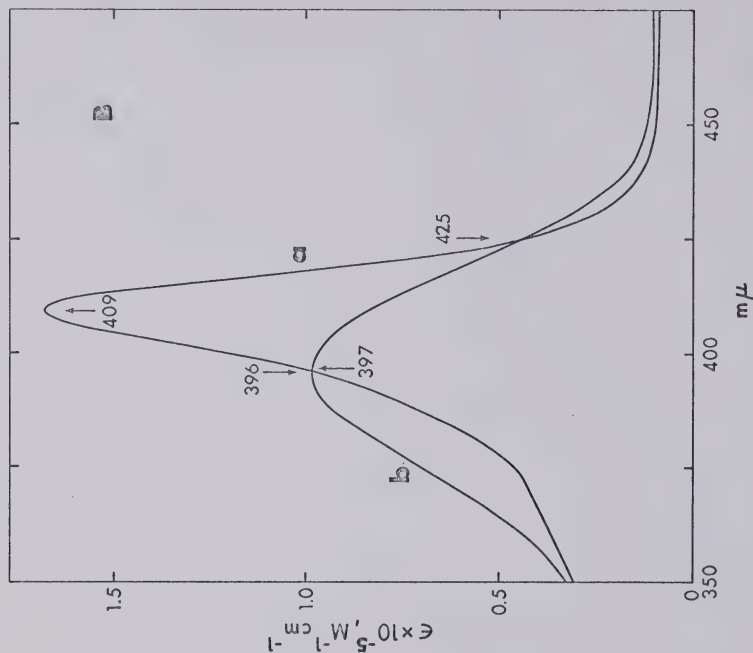
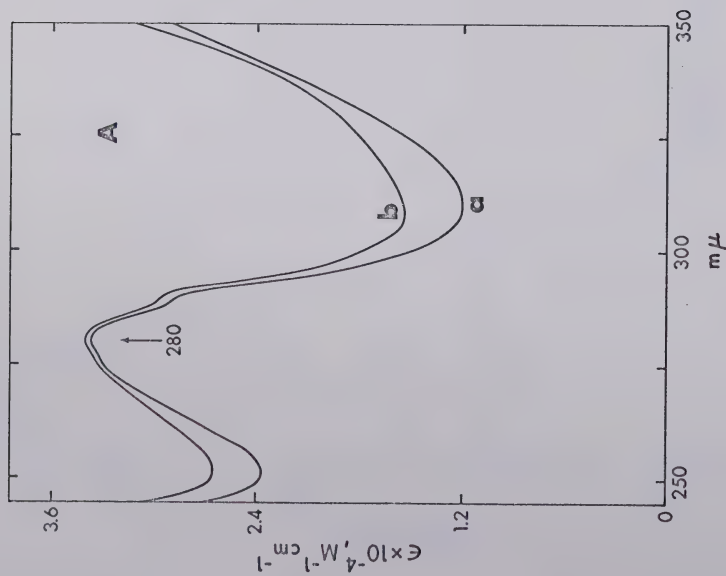


Figure 30. Ultraviolet (A) and Soret (B) absorption spectra of native (a) and BrCN-modified (b) metmyoglobin. 0.1 M phosphate, pH 7.0, 25°. A: 25 μM Mb and 490 μM BrCN. B: 5.7 μM Mb and 200 μM BrCN.

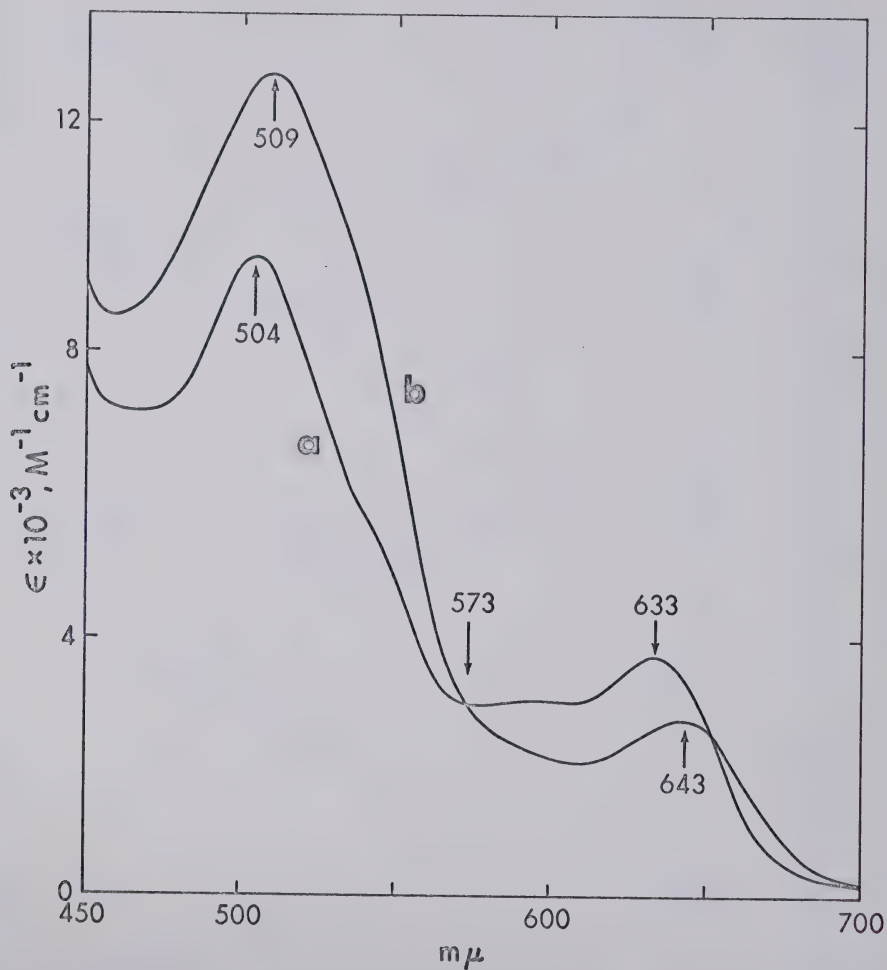


Figure 31. Visible absorption spectra of native (a) and BrCN-modified (b) metmyoglobin. 0.1 M phosphate, pH 7.0, 25°, 69 μ M MMb and 570 μ M BrCN.

TABLE XXXIX

Sedimentation Constant of Native and
BrCN-Modified Metmyoglobin

MMb Preparation	$S_{20,w} \times 10^{13}$, sec.	$S_{20,w}^{\circ} \times 10^{13}$, sec.
Native	1.68	1.76
BrCN-modified	1.69	1.77

Double sector cell, 60,000 r.p.m., $20.0 \pm 0.1^{\circ}$.

175 μ M or 0.314% MMb, 0.09 M phosphate, pH 7.0, ~1.8%
acetonitrile v/v.

Sedimentation run was performed within three hours of the
addition of 1.78 mM BrCN to obtain the BrCN-derivative.

phosphate, pH 7, 25°; the same volume of acetonitrile was added to the native metmyoglobin solution. These two solutions were run in the ultracentrifuge, and the results are given in Table XXXIX.

The identical sedimentation constants of native and BrCN-modified metmyoglobins suggest that gross structural changes do not accompany the modification of metmyoglobin by BrCN.

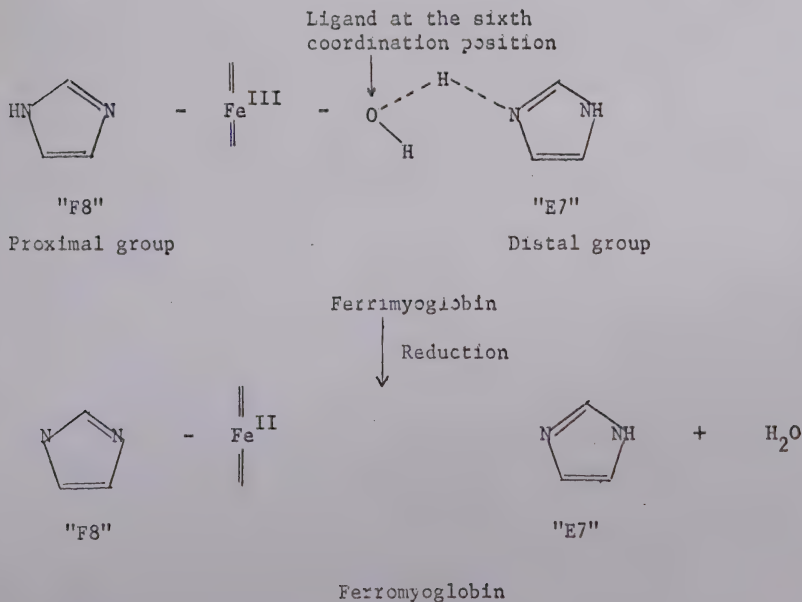
For the circular dichroism measurements, BrCN metmyoglobin was prepared by adding 250 μ M BrCN to 9 μ M metmyoglobin in 0.1 M phosphate, pH 7, 25°; the same metmyoglobin solution, without the addition of BrCN, was used as the native sample. The ultraviolet CD spectra of the two samples, shown in Fig. 29, do not indicate significant changes in the conformation of the polypeptide chain as the result of the BrCN-modification.

Absorption spectra. The ultraviolet, Soret and visible absorption spectra of native and BrCN-modified metmyoglobins are shown in Figs. 30 and 31. Under the conditions specified, metmyoglobin was stable in its BrCN-modified form during the time period required to obtain the spectra (10 - 15 min).

The spectra of the modified derivative are significantly different from those of the native protein, but do not indicate gross structural rearrangements. (See results in the previous section, and spectral properties of regenerated MMb, page 95). In the ultraviolet, the increase in extinctions could partly be contributed by the modified residue in addition to differences in hemin transitions. The latter are certainly affected as shown by the substantial changes in the

Soret and visible regions. At this stage, an interpretation of the spectroscopic features cannot be attempted, save by saying that the environment of the prosthetic group must be altered. In the simplest terms, this is taken to mean that modification of apoprotein occurred near the fifth or sixth coordination position; as a consequence, a change in hemein conformation would not be surprising.

Reduction of BrCN-metmyoglobin. In order to pinpoint the site of modification more accurately in the vicinity of heme, an experimental approach was adopted that utilized the reducibility of Fe (III) of metmyoglobin to the ferrous state. Let us also recall that in the absence of " π -ligands" such as CO, O₂, NO, CN⁻, the sixth coordination position is not occupied in ferromyoglobin (146), as is shown by the following scheme.



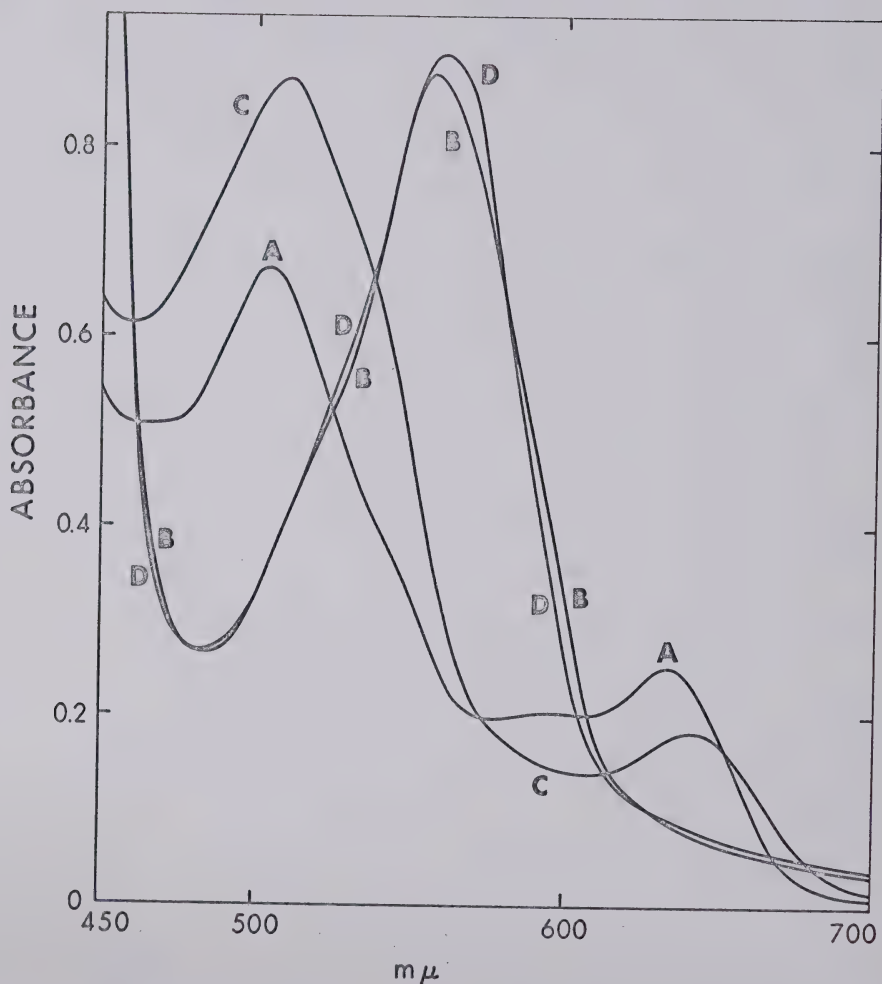


Figure 32. Visible absorption spectra of native and BrCN-modified metmyoglobin and their reduced derivatives. Curve A: native metmyoglobin; Curve B: native myoglobin; Curve C: BrCN-metmyoglobin; Curve D: BrCN-myoglobin. 0.1 M phosphate, pH 7.0, 25°, 69 μM (met)myoglobin, 570 μM BrCN. Reduction was achieved by adding minimal amounts of solid $\text{Na}_2\text{S}_2\text{O}_4$.

In addition, it has been shown that the reduction of metmyoglobin to the ferrous states does not cause any conformational changes (146); that is, the structural relationship between heme and apoprotein is the same in both forms of the protein except no water is coordinated to the heme in myoglobin, as is indicated above. Thus, it would be expected that the properties of myoglobin, for instance its absorption spectrum, should be identical for both the native and BrCN-modified molecules if the distal histidine residue is altered by its reaction with BrCN. Indeed, this is what has been found; the visible absorption spectra of the reduced forms of native and BrCN-modified metmyoglobin are almost identical, as they are shown in Fig. 32.

However, it was essential to show that upon reduction, the modification is not reversed, if any credence was to be given to the above claim. The following experiment provided such evidence. First of all, BrCN-modified metmyoglobin, obtained by mixing 480 μM MMb and 455 μM in 0.1 M phosphate, pH 7 at 25°, was reduced with a minimum amount of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$). As expected, the absorbance decreased when the reaction was observed at 525 m μ . Now, providing that the modification is not reversed after reduction of the prosthetic group, the reoxidation of the ferroporphyrin should give a corresponding increase in A_{525} . On the other hand, if on reduction the modification were reversed, then oxidation should not cause any spectroscopic change - since 525 m μ is an isosbestic point for metmyoglobin and ferromyoglobin.

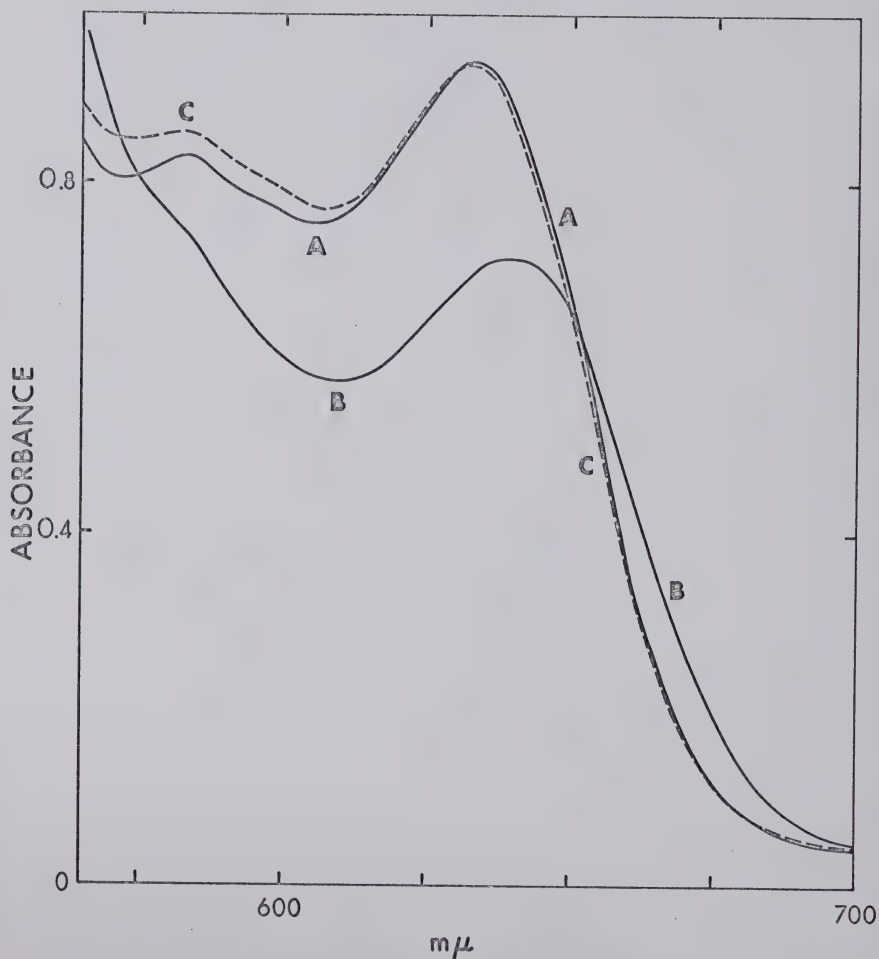


Figure 33. Visible absorption spectra of native metmyoglobin (A), BrCN-metmyoglobin (B) and regenerated metmyoglobin (C). Spectrum B was obtained 20 minutes and Spectrum C 8 hours after the addition of BrCN. 0.1 M phosphate, pH 7.0, 25°, 260 μ M MMB and 260 μ M BrCN.

TABLE XL

Molar Extinction Coefficients of Native and Regenerated
Metmyoglobins in the Soret and Visible Regions

Wavelength mμ	Native MMb $\epsilon \times 10^{-3}, M^{-1} cm^{-1}$	Regenerated MMb $\epsilon \times 10^{-3}, M^{-1} cm^{-1}$
642	3.29	3.26
633	3.62	3.58
625	6.25	6.23
600	2.92	3.06
575	2.99	3.23
550	4.64	4.93
540	5.78	5.98
504	9.05	9.12
460	6.72	6.75
420	77.5	77.0
409	167	168
390	108	107
370	40.2	39.2
350	28.8	28.4

0.1 M phosphate, pH 7, 25°.

About 10 minutes after reduction, the reoxidation was achieved by adding about 2-fold excess (over the total myoglobin hematin-Fe) ferricyanide, and as the result, the absorbance at 525 mμ increased to nearly the same initial value (the slight difference was due to regeneration). These findings clearly indicate that modification involves a group located on the distal side of the heme. The fact that the modified group is the distal histidine can be deduced from the observation that no structural changes occur within and around the heme group when metmyoglobin is reduced, except the water is lost from the distal side of the heme (see page 94). In simple terms, the only amino acid residue, whose interaction with the prosthetic group is altered in the ferric and ferrous forms of myoglobin is the distal histidine and the modification of such residue is required to explain the above observed spectroscopic characteristics of native and BrCN-modified metmyoglobins and their reduced derivatives.

Regeneration of "native-metmyoglobin" from BrCN-metmyoglobin

As was mentioned previously, BrCN-metmyoglobin, obtained by using equimolar amounts of BrCN and protein, reverts relatively rapidly to a form that is spectroscopically almost identical to native metmyoglobin. Fig. 33 shows the absorption spectra of native, BrCN-modified and regenerated metmyoglobins in the visible region. The difference between the absorption spectra of native and regenerated metmyoglobins is the greatest in the 570 to 600 mμ region, at lower wavelengths the difference diminishes again (see Table XL), and the two

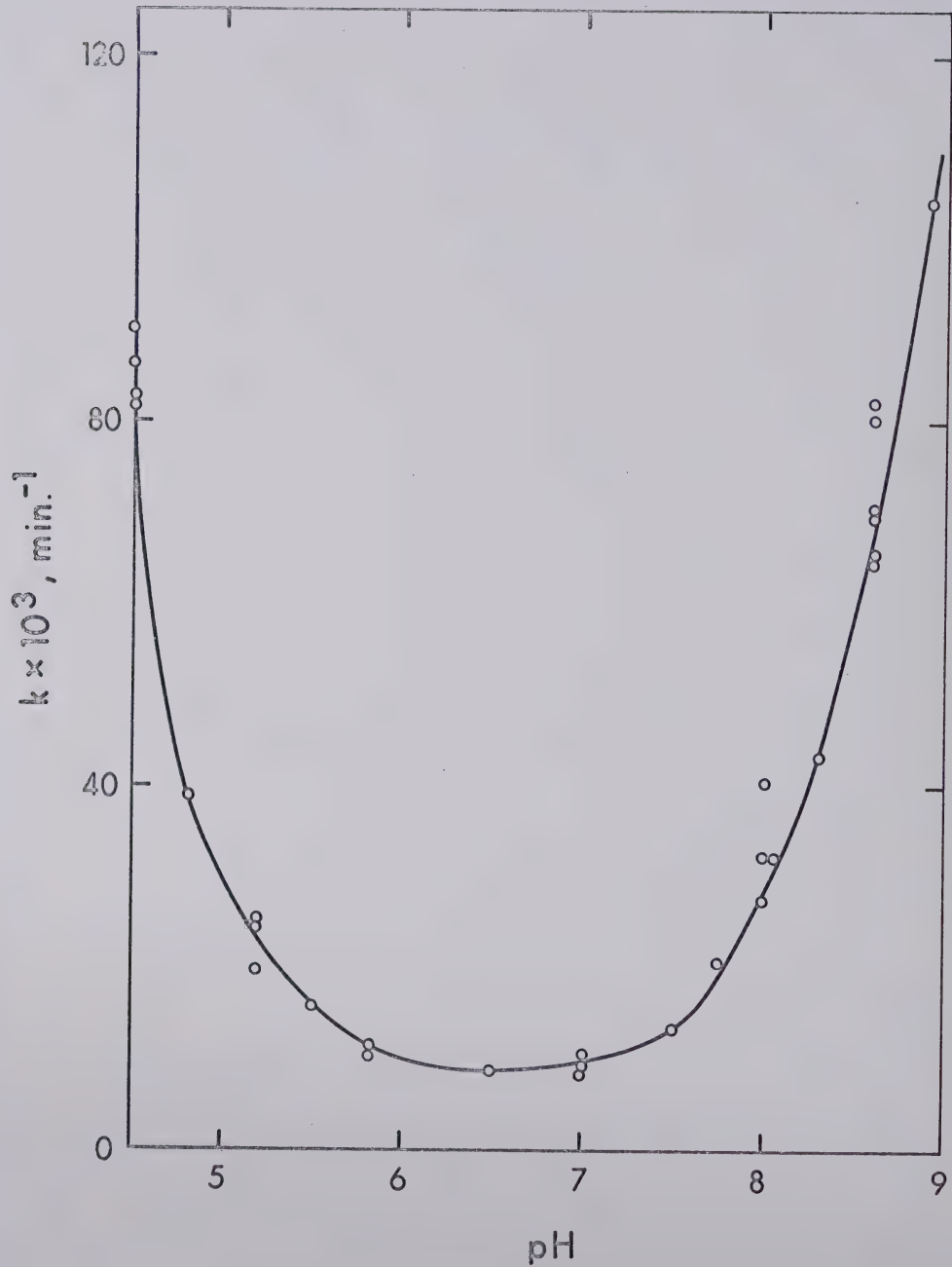


Figure 34. Effect of pH on the rate of regeneration of BrCN-metmyoglobin. Data from Table XL.

TABLE XLI

Effect of pH on the Rate of Regeneration of
Metmyoglobin from BrCN-Metmyoglobin

pH	Buffer	No. of Determinations	$k \times 10^3, \text{min.}^{-1}$
4.5	Pivalate	4	87.6
4.8	-- " --	1	39.0
5.2	-- " --	3	24.5
5.5	-- " --	1	18.0
5.8	-- " --	2	11.6
5.8	Phosphate	2	10.5
6.5	-- " --	2	8.9
7.0	-- " --	6	10.3
7.5	-- " --	2	13.6
7.8	-- " --	1	20.9
8.0	-- " --	2	29.8
8.0	Borate	2	32.4
8.3	-- " --	1	43.5
8.6	-- " --	4	75.6
8.9	-- " --	1	104

0.1 M buffers, 25°, 3.8 μM MMb.

spectra are almost identical in the Soret region as indicated by the molar extinction coefficients listed in Table XL.

The similarity of regenerated metmyoglobin to the native protein was also shown by experiments in which the inhibition-regeneration cycle was repeated up to four times using the same protein solution (in 0.1 M phosphate, pH 7, at 25°). The rate constant for the inhibition reaction became slightly lower after the second or third cycle, but they were all within the range of $30 \pm 3 \text{ M}^{-1} \text{ sec}^{-1}$ (see page 83). The rate constant for the regeneration process was the same in all cases, $10 \pm 2 \times 10^{-3} \text{ min}^{-1}$ (for comparison see Table XLI). The visible absorption spectra of the metmyoglobin solution did not show significant differences after successive regenerations, except the slight differences observed in the 570 to 600 mμ region.

The rates of metmyoglobin regeneration were determined using preformed "modified" protein, which was prepared by mixing equimolar amounts of the protein and the reagent (≈250 μM each). Fifteen minutes after initiation of MMb modification (i.e. when ≈90% of the reaction had taken place), aliquots of modified protein were diluted in appropriate buffers and the rate of regeneration, characterized by absorbance increase at 410 mμ, was determined at 25°. The results, given in Table XLI and Fig. 34, establish acceleration of activation reaction under acidic, or basic conditions - a property which is most readily reconciled with the behaviour expected for N-cyanomidazole. The latter compound hydrolyses in alkaline solution, the second order constant being approximately, $k = 1.3 (\pm .1) \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$ (49);

TABLE XLII

Acid Hydrolysis of BrCN-MMb

Exp. No.	[MMb], μM	[Br ¹⁴ CN], μM	Amount of ¹⁴ C, d.p.m.	Amount of ¹⁴ C found ^b			
				in MMb solution		in NaOH solution	
				d.p.m.	%	d.p.m.	%
1	490	470	8,400,000	260,000	3	8,150,000	97
2	490	470	8,400,000	650,000	8	7,500,000	90
3	490	412	7,600,000	480,000	6	7,200,000	95
						163,000	2
						225,000	3
						216,000	3

Glass distilled water, pH ~ 6.5 , 25°. For inhibition reaction: $t=1/2 = \sim 1$ min.

The pH of the MMb solution was lowered to ~ 1.2 (with conc. HCl) about 10 min. after the addition of Br¹⁴CN.

^a Estimated by removing a sample right after Br¹⁴CN addition.

^b Estimated after 18 hours of incubation at $\sim 25^\circ$.

^c After addition of ~ 0.25 M BaCl₂ to the NaOH solution.

this compares with $1.1 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$ for the solvolysis of cyanated metmyoglobin. Equally, in neutral media, the relative rate constants are $k_{\text{MMb-CN}}/k_{\text{N-CN imidazole}} \sim 0.7$. But, under acidic conditions, the analogies break down, possibly due to the formation of N-carbamyl imidazole which seems to be formed preferentially in the model system (49).

Acid hydrolysis of Br^{14}CN -metmyoglobin. In an attempt to establish the nature of product released when BrCN -metmyoglobin is regenerated the experiments that were performed with Br^{14}CN catalase were repeated here using Br^{14}CN -metmyoglobin.

The apparatus utilized here was the same that was described in connection with the catalase experiments (see page 69). $490 \mu\text{M}$ metmyoglobin was in one of the vessels and 0.1 M NaOH in the other. Less than stoichiometric amount of BrCN (412 or $470 \mu\text{M}$) was added to the protein solution; after mixing a sample was removed to estimate the amount of the radioactivity present. About 10 minutes after the addition of Br^{14}CN , a predetermined volume of concentrated HCl was added to the protein solution to lower the pH to ~ 1.2 ; the apparatus was closed and the solutions were incubated for 18 hours at room temperature. After the incubation, the radioactivity was estimated in both solutions, and then barium chloride was added to the NaOH solution (final $[\text{BaCl}_2] = \sim 0.02\text{M}$). After the precipitate settled out, the radioactivity present in the supernatant was determined. The results of these experiments are given in Table XLII.

The experimental evidence obtained in these experiments suggests, as in the case of catalase (see page 69), that the modified residue is hydrolyzed under acidic conditions and HO^{14}CN is released. In a subsequent reaction, cyanic acid is hydrolyzed to carbon dioxide and ammonia and the resultant $^{14}\text{CO}_2$ is trapped in the NaOH solution, as it is indicated by its precipitation by BaCl_2 .

It has to be mentioned here that the release of ^{14}C from Br^{14}CN -metmyoglobin under acidic conditions is another piece of evidence that implicates histidine as the site modification. It was pointed out earlier that on the distal side of the heme in metmyoglobin only a histidine (E7) and a threonine (C4) could be considered as the sites of modification (see page 75). The release of ^{14}C under acidic conditions eliminates threonine as the site of modification, because it has been shown that alkyl cyanates, R-O-CN add water to form carbamates, R-CO-NH_2 , which are resistant to further hydrolytic attack even in concentrated HCl (168). Thus, the modification of the distal histidine residue by BrCN is indicated again.

ConclusionsActivation of cyanogen bromide

It is only seldom that a high specificity can be achieved in chemical modification of proteins. Whenever it is achieved, the inhibitor, almost invariably, has some structural features which typify the substrate; hence the basis of a modern approach to chemotherapy.

Another interpretation of the specific protein modification rests on a premise that some amino acid functional group exhibit an unusual reactivity - but the reference standard for the 'unusual' has only too wide a latitude. Generally, it is taken to mean: greater reactivity relative to other, chemically identical, residues within the same protein matrix; but whether this represents 'masked' reactivity of other groups or expresses a coincidental binding of a modifier near the potentially reactive site is, more often than not, a matter of semantics. The situation is rather different when it can be shown that the group undergoing modification plays an essential catalytic role. For then, it becomes possible that its reactivity is a reflection of a unique structural constellation - the active site - whose elucidation is one of the prime aims in enzymology.

The reaction of 3-amino-1,2,4-triazole with catalase compound I (93) (see p. 13) was conceived in these terms; and it implied the formation of a uniquely reactive histidine (HIS-74) following interaction of catalase and hydrogen peroxide (97). The functional significance of such an activation to the catalytic process were not defined, but the suggested susceptibility of HIS-74 to nucleophilic attack by amino

triazole has important consequences. It can mean - but was not stated in these terms - that formation of compound I is attended by oxidation of an imidazole group whose reduction can be achieved either peroxidatically (with e.g. ethanol), catalatically (with H_2O_2) or irreversibly (with aminotriazole).

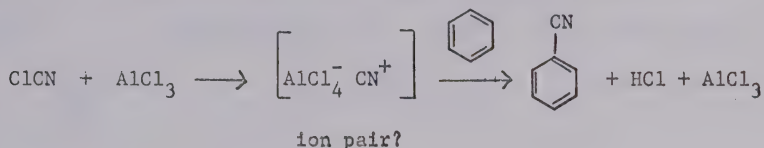
An opposite interpretation (p. 14) is to invoke oxidation of amino-triazole giving an electrophilic reagent which then rapidly combines with a distal histidine. In this sense, it is an example of a 'cage effect' so frequently encountered in free radical chemistry. A similar argument holds for cyanogen bromide - catalase reaction; it will be given presently. But, first of all, note that the inhibited catalases have almost identical physico-chemical properties irrespective of their mode of formation - via BrCN reaction (in the absence of H_2O_2) or in the aminotriazole - compound I system.

Both reactions entail the modification of a single group; both derivatives have similar spectra; both show the same response, or lack of it, towards various potential catalase ligands. Given these similarities, it seemed likely that either reaction is centered at the same locus. However, in the cyanogen bromide pathway, there is no apparent reason to postulate activation of histidine. No compound I is present; nor is the derivative reducible. Thus to explain the highly localized action of BrCN one is led to consider the possibility of its activation.

Polarization of cyanogen bromide

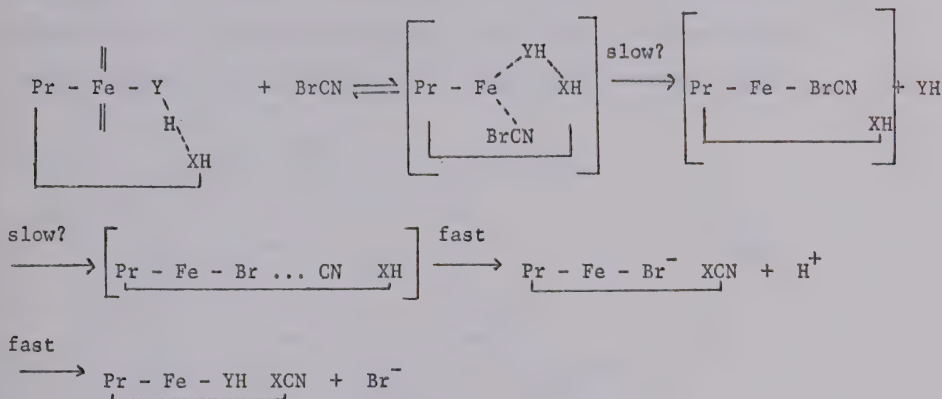
Binding of BrCN to hemin, or more specifically, interaction of bromine end of the molecule with a positively charged metal ion (Fe^+) should result in a distortion of the electronic distribution of

cyanogen bromide. Numerous examples come to mind to illustrate this principle, for instance : ferric chloride catalyzes the aromatic iodination by ICl (149); carbonium ions can be generated from alkyl halides in the presence of silver salts (150); compounds containing an active methylene group react with pentacyanonitrosyl ferrate (II) to give oximes (151) and activation of acyl halides by Lewis acids is an integral part of Friedel-Crafts acylation. A typical Friedel-Crafts catalyst, AlCl_3 , can be used also to activate cyanogen chloride which then reacts with benzene to form benzonitrile (152):



The type of bonding the ligand (BrCN) and the Lewis acid catalyst (ferriprotoporphyrin) is more difficult to specify. The extent of σ - type coordinate bond formation will depend on the differences in the electronegativity of the bonded molecules. In addition, there is a possibility of π -bonding. Here, in contrast to σ - type bond, the effect on ligand polarization is less predictable. If it involves considerable electron donation from (filled) d_{xz} , d_{xy} or d_{yz} orbitals of the metal to (empty) molecular orbitals of the ligand, some depolarization of the ligand will take place. In a case of strong overlap, the potential electrophile would be actually stabilized relative to its reactivity in a free state, or even reduced by electron transfer from the metal ion. Of course, π - bonding does not operate necessarily in opposition to σ -type polarization; it can in fact reinforce it if p-electrons of the ligands were to be shared with suitable empty orbitals of the metal ion. Therein must lie, at least, partial answers to the hemoproteins versatility and here we look for

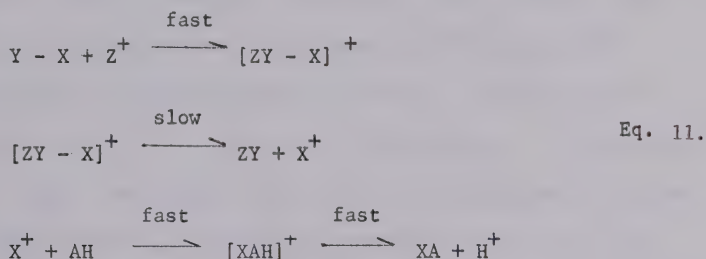
the extraordinary specificity of cyanogen bromide reaction. In short, it will be assumed that reaction involves binding of cyanogen bromide to ferriprotoporphyrin, probably necessitating displacement of the sixth coordination ligand present in the 'native complex'. In this configuration, ionization of BrCN is induced, giving CN^+ which then combines rapidly with an adjacent nucleophile, hopefully, a histidine residue:



No build up of the enzyme-cyanogen bromide complex must occur; otherwise, the kinetics would lose their bimolecular character. And to account for pH-invariance of inactivation, it becomes essential to postulate that generation of CN^+ takes place in a rate-limiting step, but whether this represents ligand interchange or a scission of BrCN bond remains unknown. The latter is perhaps not an only factor because if this were so, then both metmyoglobin and catalase reactions would occur at identical rates. The observed some four-fold difference (Table XV, and page 83) in the 'inactivation rate constants'

is indeed small and could well indicate differences in the extent of σ - or π - type polarization.

The lack of pH dependence observed in both metmyoglobin and catalase inactivations is now a necessary corollary. It is analogous to the rate-determining formation of nitronium ion, or bromonium ion, in nitrations, or bromination of some homocyclic compounds (153). Similar observations hold for heteroaromatic systems, in the case of diazocoupling of indole and imidazole (154,155). All these cases share a common feature, which can be summarized according to Eq. 11.



where $\text{Y} - \text{X}$ is an electrophile

Z^+ an activating species (e.g. H_3O^+)

and AH a reactive acceptor molecule.

Only when the rate of X^+ generation is greater than its combination with AH can we expect dependence on the concentration of the acceptor. Referring to the cyanation reaction, this means that the rate of CN^+ formation must be smaller than the rate of combination with the 'imidazole' nucleus. No matter how small, or how large, the effective concentration of the acceptor, this will not be observable in the kinetic analysis unless the rate of 'CN' dissipation were to become limiting.

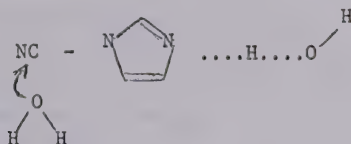
Regarding the relative stability of 'cyanated' metmyoglobin and catalase, even more speculative remarks can be offered.

Stability of the modified derivatives

Three concepts could be developed in attempting to give some explanation of the marked differences in the stabilities of metmyoglobin and catalase derivatives. The first, rather obvious, would require that modification does not involve the same functional groups. Examination of the crystal structure of metmyoglobin does not provide a ready alternative to histidine. With catalase, the situation is more complex but since Agrawal and Margoliash (97) found some 70% of trapped aminotriazole within a single peptide - and identified the locus of reaction to be histidine - it is difficult to reconcile this result with the presence of several reactive groups in the 'distal' cavity. Moreover, assuming that such groups are present, it would follow that cyanation of any one group gives equal enzyme inhibition; this requirement is imposed by the relationship between stoichiometry of cyanation and loss of enzyme activity (p. 64). Furthermore, such random modifications would have to occur at groups capable of releasing only a single proton (tyrosine; serine; threonine or cysteine); all would have equal stabilities between pH 4 and 9; all would be labile on enzyme denaturation and give carbon dioxide in almost a quantitative yield. Surely, this is not the most attractive scheme.

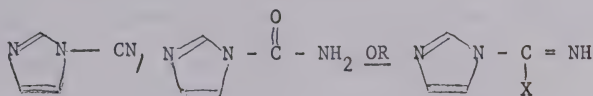
The second possibility is a 'steric' or environmental hypothesis. It demands a different accessibility of water to the site of modification or could be taken to mean that a general acid - nucleophilic catalysis, necessary for de-cyanation, cannot occur with equal facility

in the two cases. Thus, the conjugate base of imidazole is a poor leaving group and its expulsion would be aided by a suitable juxtaposition of a general acid catalyst (HX), for instance - water:



A possible test of this scheme would be to study reactivation in D_2O .

In the third scheme we would also postulate modification of the same residue, but in a different chemical constellation, as would be the case for derivatives such as:



The evaluation of these hypotheses is the task for the future, as is the extension of pseudohalogen reactions to other hemoprotein systems.

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APPENDIX

Calculation of rate constants from kinetic measurements*.

1. First order conditions.

Let us consider the reaction: $a + b \longrightarrow \text{products}$.

When $b \gg a$, b is essentially constant during the reaction, and therefore: $\text{rate} = k^1[a]$, where $k' = k[b]$, and first order kinetics are observed, that is the system obeys the integrated form of the first order rate law: $k't = 2.303 \log \frac{a}{a-x}$

a) Graphical method

When there is a difference in absorbances of the reactants and products, the reaction can be followed spectrophotometrically. Let us say that the initial absorbance value A_0 decreases to the final value of A_∞ as the reaction goes to completion. Furthermore, let us suppose that ϵ is the molar extinction coefficient of one of the reactants and $\Delta\epsilon$ is the change in the molar extinction coefficient as the result of the reaction. Then

$$a = \frac{A_0}{\epsilon} \quad \Delta\epsilon = \frac{\Delta A}{a} = \frac{A_0 - A_\infty}{\frac{A_0}{\epsilon}} = \frac{(A_0 - A_\infty)\epsilon}{A_0}$$

At any time t during the reaction the concentration of the absorbing reactant will be $a - x$, where x is the amount of a converted to product(s). From the above definitions

$$x = \frac{A_0 - A_t}{\Delta\epsilon} = \frac{(A_0 - A_t)A_0}{(A_0 - A_\infty)\epsilon}$$

Substituting for the values of a and x , we get:

$$\frac{a}{a-x} = \frac{\frac{A_0}{\epsilon}}{\frac{A_0}{\epsilon} - \frac{(A_0 - A_t)A_0}{(A_0 - A_\infty)\epsilon}}$$

Multiplying both the numerator and the denominator by $\frac{\epsilon(A_0 - A_\infty)}{A_0}$

we get

$$\frac{a}{a-x} = \frac{A_0 - A_\infty}{A_0 - A_\infty - (A_0 - A_t)} = \frac{A_0 - A_\infty}{A_t - A_\infty + A_0 - A_0} = \frac{A_0 - A_\infty}{A_t - A_\infty}$$

Substituting this into the integrated rate equation we get:

$$k't = 2.303 \log \frac{A_0 - A_\infty}{A_t - A_\infty}$$

Rearranging, we have $k't = 2.303 \log (A_0 - A_\infty) - 2.303 \log (A_t - A_\infty)$.

In this equation the first term on the right hand side is constant, therefore if we plot $\log (A_t - A_\infty)$ against time we should obtain a straight line. The slope of this line is $-\frac{k'}{2.303}$, and thus k' can be conveniently calculated. From the first order rate constant, k' , the second order rate constant can be calculated:

$$k = \frac{k'}{[b]}$$

b) $t=1/2$ method

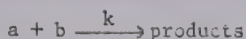
A much simpler method to calculate k' from a kinetic experiment. Under the conditions described above $t=1/2$ is the time required for the reaction to go 50% completion. From this value k' can be calculated by using the following equation:

$$k' = \frac{0.693}{t-1/2}$$

This equation is derived from the integrated form of the first order rate law by substituting $x = a/2$ and $t = t-1/2$.

2. Second order conditions

If in the reaction



the concentrations of a and b are equal or nearly equal, second order kinetics will be observed. For second order kinetics two cases have to be discussed; the special case when the concentrations of a and b are equal, and the general case when they are different. The definition of A_0 , A_t , A_∞ , ε , and $\Delta\varepsilon$ are as given under first order conditions.

a) Special case of second order kinetics

1. Graphical method

When the concentrations of a and b are equal the integrated second order rate equation takes the following form:

$$kt = \frac{x}{a(a-x)}$$

where x denotes the decrease in concentration of both a and b.

$$\text{Now, } a = \frac{A_0}{\varepsilon}, \quad x = \frac{A_0 - A_t}{\Delta\varepsilon}, \quad \Delta\varepsilon = \frac{A_0 - A_\infty}{a} = \frac{(A_0 - A_\infty)\varepsilon}{A_0}$$

$$\text{Then, } x = \frac{(A_0 - A_t)A_0}{(A_0 - A_\infty)\varepsilon}$$

Substituting for a and x, we get

$$akt = \frac{\frac{(A_0 - A_t)A_0}{(A_0 - A_\infty)\varepsilon}}{\frac{A_0}{\varepsilon} - \frac{(A_0 - A_t)A_0}{(A_0 - A_\infty)\varepsilon}}$$

Multiplying both the numerator and the denominator by the factor $\frac{(A_0 - A_\infty)\varepsilon}{A_0}$, we get

$$akt = \frac{(A_0 - A_t)}{(A_0 - A_\infty) - (A_0 - A_t)} = \frac{A_0 - A_t}{A_t - A_\infty}$$

$$\text{Since } A_0 - A_t = (A_0 - A_\infty) - (A_t - A_\infty)$$

$$\text{then } kt = \frac{(A_0 - A_\infty) - (A_t - A_\infty)}{a(A_t - A_\infty)} = -\frac{1}{a} + \frac{(A_0 - A_\infty)}{a(A_t - A_\infty)}$$

$$\text{For } a \text{ we can substitute } \frac{A_0 - A_\infty}{\Delta \varepsilon}$$

$$kt = -\frac{1}{a} + \frac{(A_0 - A_\infty) \Delta \varepsilon}{(A_0 - A_\infty) (A_t - A_\infty)}$$

$$kt = -\frac{1}{a} + \frac{\Delta \varepsilon}{(A_t - A_\infty)}$$

$$\frac{kt}{\Delta \varepsilon} + \frac{1}{a \Delta \varepsilon} = \frac{1}{(A_t - A_\infty)}$$

By plotting $\frac{1}{(A_t - A_\infty)}$ against t we get a straight line.

The slope of the line will be $k/\Delta \varepsilon$ and y intercept $1/a \cdot \Delta \varepsilon$.

Therefore $k = \text{Slope} \times \Delta \varepsilon$.

ii. $t-1/2$ method

From the $t-1/2$ the rate constant can be calculated by the following equation:

$$k = \frac{1}{t-1/2 \cdot a}$$

b) General case of second order kinetics*

i. Graphical method

In this case $a \neq b$, and the integrated form of the second order rate equation is as follows:

$$kt = \frac{2.303}{a-b} \log \frac{b(a-x)}{a(b-x)}$$

Let us define $a > b$, and $n = \frac{a}{b}$ or $a = nb$.

$$\text{Then (1) } \frac{2.303}{a-b} = \frac{2.303}{nb-b} = \frac{2.303}{b(n-1)}$$

$$\begin{aligned} (2) \quad \log \frac{b(a-x)}{a(b-x)} &= \log \frac{b(nb-x)}{nb(b-x)} = \log \frac{1}{n} + \\ &+ \log \frac{(nb-x)}{(b-x)} \end{aligned}$$

$$(3) \quad (nb-x) = (n-1)b + (b-x)$$

Substituting (3) into (2) we get

$$\log \frac{1}{n} + \log \frac{(nb-x)}{(b-x)} = \log \frac{1}{n} + \log \frac{(n-1)b + (b-x)}{(b-x)}$$

$$\begin{aligned} (4) \quad \log \frac{1}{n} + \log \frac{(nb-x)}{(b-x)} &= \log \frac{1}{n} + \\ &+ \log \left[1 + \frac{(n-1)b}{b-x} \right] \end{aligned}$$

$$\text{Let } b = A_o - A_{\infty} \quad \text{and} \quad x = A_o - A_t$$

$$\text{Then } b-x = A_o - A_{\infty} - A_o + A_t = A_t - A_{\infty}$$

Substituting these into (4) we obtain:

$$\begin{aligned} (5) \quad \log \frac{1}{n} + \log \left[1 + \frac{(n-1)b}{b-x} \right] &= \log \frac{1}{n} + \\ &+ \log \left[1 + \frac{(n-1)(A_o - A_{\infty})}{A_t - A_{\infty}} \right] \end{aligned}$$

Now substituting expressions (1) and (5) into the rate equation we obtain:

$$kt = \frac{2.303}{(n-1)b} \left(\log \frac{1}{n} + \log \left[1 + \frac{(n-1)(A_o - A_{\infty})}{A_t - A_{\infty}} \right] \right)$$

$$\text{Let } A = \frac{2.303}{(n-1)b} \quad \text{and} \quad B = (n-1)(A_o - A_{\infty})$$

$$\begin{aligned}\text{Then } kt &= A \left[\log \frac{1}{n} + \log \left(1 + \frac{B}{A_t - A_\infty} \right) \right] \\ &= A \log \frac{1}{n} + A \log \left(1 + \frac{B}{A_t - A_\infty} \right)\end{aligned}$$

Substituting $C = A \log \frac{1}{n}$ we get:

$$kt = C + A \log \left(1 + \frac{B}{A_t - A_\infty} \right)$$

By plotting $\log \left(1 + \frac{B}{A_t - A_\infty} \right)$ against t we get a straight line with slope k/A and a 'y' intercept of C .

Therefore $k = \text{Slope} \cdot A$

ii. $t-1/2$ method

Let a , b , n , be defined as above. In addition let us define the time periods: t_1 , t_2 , t_3 , and so on as follows:

$$t_1 = \text{first } t-1/2, \quad t_2 = \text{first } t-1/2 + \text{second } t-1/2,$$

$$t_3 = \text{first } t-1/2 + \text{second } t-1/2 + \text{third } t-1/2$$

and so on.

At the end of the time period t_1 b has decreased to half of its original value and therefore x will be $b/2$.

In the second order rate equation:

$$kt = \frac{2.303}{a-b} \log \frac{b(a-x)}{a(b-x)}$$

we can substitute in the following manner:

$$(a-b) = bn - b = b(n-1)$$

$$b(a - x) = b(nb - \frac{b}{2}) = b^2(n - 1/2)$$

$$a(b - x) = nb(b - \frac{b}{2}) = \frac{nb^2}{2}$$

Putting these terms back into the rate equation we get

$$kt_1 = \frac{2.303}{b(n-1)} \log \frac{b^2(n-1/2)}{\frac{nb^2}{2}} = \frac{2.303}{b(n-1)} \log \frac{2n-1}{n}$$

In similar manner equations can be obtained for conditions prevailing at the end of time periods: t_2 , t_3 , t_4 , and so on. All these relationships are described by the following general equation:

$$k = \frac{1}{t_1} \cdot \frac{2.303}{b(n-1)} \log \frac{2^i(n-1) + 1}{n}$$

where $i = 1, 2, 3, 4, \dots$, corresponding to the respective time periods $t_1, t_2, t_3, t_4, \dots$.

From the above equation the rate constant can be easily calculated.

*

As general reference, refer to A. A. Frost and R. G. Pearson, "Kinetics and Mechanisms", 2nd Edition, John Wiley and Sons, Inc., New York, 1961.

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With respect to 2. (b) - General case of second order kinetics, refer to M. L. Bender, G. R. Schonbaum, and B. Zerner, J. Am. Chem. Soc., 84, 2562 (1962).

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